09.27.00 A

JC820 U.S. PTO 09/25/00

EXPRESS MAIL CERTIFICATE

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Typed or Printed Name	Wilhelm Albert Palmen Jr.	**Express Mail No.**	EL 563 386 315 US
Signature	Billy Palmen	**Date**	September 25, 2000

JC813 U.S. PTO 09/669033 09/25/00

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Atty Docket No.	OLIG-017CON
First Named Inventor	Roderic M.K. Dale
Title:	"METHOD FOR DETECTING NUCLEIC ACID SEQUENCES"

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents

Address to: Assistant Commissioner for Patents
Box Patent Application
Washington, D.C. 20231

1. X Fee Transmittal Form

2. X Specification Total Pages 48
(preferred arrangement set forth below)
- Descriptive title of the invention
- Cross Reference to Related Applications
- Statement Regarding Fed sponsored R & D
- Reference to Microfiche Appendix
- Background of the Invention
- Brief Summary of the Invention
- Brief Description of the Drawings (if filed)
- Detailed Description
- Claim(s)
- Abstract of the Disclosure

3. X Drawing(s) *(35 USC 113)* Total Sheets 9

4. X Oath or Declaration Total Sheets 2

a. __ Newly executed (original or copy)
b. X Copy from a prior application (37 CFR 1.63(d) (for continuation/divisional with Box 16 completed)

i. __ DELETION OF INVENTOR(S)
Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b)

c. __ Unsigned

5. __ Microfiche Computer Program (*Appendix*)

6. __ Nucleotide and/or Amino Acid Sequence Submission (*if applicable, all necessary*)

a. __ Computer Readable Copy
b. __ Paper Copy (identical to computer copy)
c. __ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

7. __ Assignment Papers (cover sheet & document(s))

8. __ 37 CFR 3.73(b) Statement __ Power of Attorney
(when there is an assignee)

9. __ English Translation Document *(if applicable)*

10. __ Information Disclosure Statement (IDS)/PTO-1449 __ Copies of IDS Citations

11. X Preliminary Amendment

12. X Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)

13. __ Small Entity Statement(s) X Statement filed in prior application Status still proper and desired

14. __ Certified Copy of Priority Document(s)
(if foreign priority is claimed)

15. X Other: Application Cover Page

16. If a CONTINUING APPLICATION, *check appropriate box and supply the requisite information:*

X Continuation of prior application No. 09/385,796 filed August 30, 1999

UTILITY PATENT APPLICATION TRANSMITTAL
(Only for new non-provisional applications under 37 CFR 1.53(b))

17. CORRESPONDENCE ADDRESS			
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SIGNATURE of Applicant or Assignee of Record	
Individual Name	Dianna L. DeVore
Registration No.	42,484
Signature	Dianna L DeVore
Date	September 25, 2000

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FEE TRANSMITTAL

Note: Effective October 1, 1998.
Patent fees are subject to annual revision.

Attorney Docket Number	OLIG-017CON
First Named Inventor	Roderic M.K. Dale
Application Number	To Be Assigned
Filing Date	Herewith (September 25, 2000)
Group Art Unit	Unassigned
Examiner Name	Unassigned

jc813 U.S. PTO 09/669033 09/25/00

METHOD OF PAYMENT

1. ___ A Check Is Enclosed
2. X The Commissioner is hereby authorized to charge the following and any additional fees including fees required under 37 CFR 1.16 and 1.17 and credit any overpayments to: Deposit Account No. 50-0815.
   Deposit Account Name: Bozicevic, Field & Francis LLP

FEE CALCULATION

1. FILING FEE

Large Fee Code	Entity Fee ($)	Small Fee Code	Entity Fee ($)	Fee Description	Fee Due
101	690	201	345	Utility filing fee	$345.00
102	310	206	155	Design filing fee	
104	480	207	240	Plant filing fee	
109	690	208	345	Reissue filing fee	
110	150	214	75	Provisional filing fee	
				Subtotal (1)	$345.00

2. CLAIMS

No. of claims as filed or after amendment			Most claims previously paid		Extra claims		Fee from below		Fee Due
Total claims	9	-	20	=	0	x	9.00	=	$0.00
Ind. claims	3	-	3	=	0	x	39.00	=	$0.00
Multiple Dependent claims						x		=	

Large Fee Code	Entity Fee ($)	Small Fee Code	Entity Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	78	202	39	Independent claims in excess of 3
104	260	204	130	Multiple dependent claim
109	78	209	39	Reissue independent claims over original patent
110	18	210	9	Reissue claims in excess of and over original patent

Subtotal (2)$0.00

3. ADDITIONAL FEES

Large Fee Code	Entity Fee ($)	Small Fee Code	Entity Fee ($)	Fee Description	Fee Due	Large Fee Code	Entity Fee ($)	Small Fee Code	Entity Fee ($)	Fee Description	Fee Due
105	130	205	65	Surcharge - late filing fee or oath		127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
139	130	139	130	Non-English specification		147	2,520	147	2,520	Filing a request for reexamination	
115	110	215	55	Ext. for reply within first month		116	380	216	190	Ext. for reply within second month	
117	870	217	435	Ext. for reply within third month		118	1,360	218	680	Ext. for reply within fourth month	
128	1,850	228	925	Ext. for reply within fifth month		119	300	219	150	Notice of Appeal	
120	300	220	150	Filing brief in support of appeal		121	260	221	130	Request for oral hearing	
140	110	240	55	Petition to revive - unavoidable		141	1,210	241	605	Petition to revive - unintentional	
142	1,210	242	605	Utility issue fee (or reissue)		122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Petitions related to prov. appl.		126	240	126	240	Submission of IDS	
146	690	246	345	Filing submission after final rejection (37 CFR 1.129(a))		149	690	249	345	For each additional invention to be examined (37 CFR 1.129(b))	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action		113	1840*	113	1840*	Requesting publication of SIR after Examiner action	
143	430	243	215	Design issue fee		144	580	244	290	Plant issue fee	
581	40	581	40	Recording patent assignment		Other fee (specify)					

* Reduced by Basic Filing Fee Paid

SUBTOTAL (3)$0.00

TOTAL AMOUNT TO BE CHARGED ($)345.00

SUBMITTED BY				Complete (if applicable)	
Typed or Printed Name	Dianna L. DeVore, BOZICEVIC, FIELD & FRANCIS LLP			Reg. Number	42,484
Signature	Dianna L DeVore	Date	September 25, 2000	Deposit Account	50-0815

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Typed or Printed Name	Wilhelm Albert Palmen Jr.	**Express Mail No.**	EL 563 386 315 US
Signature		**Date**	September 25, 2000

PRELIMINARY AMENDMENT Address to: Assistant Commissioner for Patents Washington, D.C. 20231	Attorney Docket	OLIG-017CON
	First Named	Roderic M.K. Dale
	Application Number	To Be Assigned
	Filing Date	Herewith (September 25, 2000)
	Group Art Unit	Unassigned
	Examiner Name	Unassigned
	Title	METHOD FOR DETECTING NUCLEIC ACID SEQUENCES

Sir:

This is a preliminary amendment to the patent application identified above. Prior to examination of the subject application, please enter the following amendments to the specification and claims:

In the Specification:

On page 1, before "FIELD OF THE INVENTION", please insert the following:

--CROSS-REFERENCE

This application is a continuation application of Serial No. 09/385,796, filed August 30, 1999, which is incorporated herein by reference in its entirety and to which we claim priority under 35 USC §120.--

In the Claims:

Please cancel original claims 1-5 and add the following new claims 6-

6. (New) A method for detecting nucleic acid sequences in two or more collections of nucleic acid molecules, the method comprising:

(a) providing an array of modified polynucleotides bound to a solid surface, each said modified polynucleotide comprising a determinable nucleic acid;

(b) contacting the array of modified polynucleotides with a first collection of labeled nucleic acid comprising a sequence substantially complementary to a nucleic acid of said array, and

detecting hybridization of the first collection of labeled complementary nucleic acids to nucleic acids of said arrays;

(c) removing said hybridized nucleic acids;

(d) contacting said array with a second collection of labeled nucleic acid comprising a sequence substantially complementary to a modified polynucleotide of said array; and

(c) detecting hybridization of the first and second labeled complementary nucleic acids to nucleic acids of said arrays;

wherein the modified oligonucleotides are characterized by a pH stability of at least one hour at 37°C at a pH in a range of about 0.5 to 6 and a nuclease resistance of at least twice that of a naturally occurring oligonucleotide having the same sequence and number of bases.

7.(New) The method of claim 6, wherein the first and second collections of nucleic acids are differentially labeled.

8.(New) The method of claim 6, wherein the first and second collections of nucleic acids comprise the same detectable label.

9.(New) The method of claim 6, wherein the step of removing said hybridized nucleic acids comprises incubation of the array with pH 1-2 acid solution.

10. (New) The method of claim 6, wherein the step of removing said hybridized nucleic acids comprises incubation incubation of the array with nuclease.

11.(New) A method of identifying nucleotide differences between the sequence of a target nucleic acid and the sequence of a reference nucleic acid comprising:

a) providing a substrate comprising different modified polynucleotide probes of known sequence at known locations;

b) contacting the target nucleic acid with the modified polynucleotide probes attached to the substrate under conditions for high specificity complementary hybridization;

c) determining which modified polynucleotide probes have hybridized with the target

nucleic acid;

d) removing hybridized target nucleic acid from the polynucleotide probes;

e) contacting the reference nucleic acid with the modified polynucleotide probes attached to the substrate under conditions for high specificity complementary hybridization; and

f) comparing the sequence of the reference nucleic acid with the sequences of the modified polynucleotide probes that have hybridized with the target nucleic acid and to identify the nucleotide differences between the sequence of the target nucleic acid and the sequence of the reference nucleic acid.

12.(New) The method of claim 11, wherein each of the different modified polynucleotide probes is attached to the surface of the substrate in a different predefined region.

13.(New) The method of claim 12, wherein each of the modified polynucleotide probes in a predefined region has a different determinable sequence, and further wherein each probe is at least 4 nucleotides in length.

14. (New) The further wherein the modified oligonucleotides are characterized by a pH stability of at least one hour at 37°C at a pH in a range of about 0.5 to 10; and a nuclease resistance of at least twice that of a naturally occurring oligonucleotide having the same sequence and number of bases.

REMARKS

Claims 6-14 are now pending in this application.

Claims 1-5 have been canceled from the application and new claims 6-14 have been added in order to more particularly point out and distinctly claim the invention. The newly added claims are fully supported within the original application. No new matter has been added.

In order to point out specific support for the claims, reference is made to the specification and the original claims as filed application Serial No. 09/385,796. Specific support can be found as follows:

Support for claim 6 and 7 can be found in originally filed claim 1 and in the specification at page 8, line 28 through page 9, line 11.

Support for claim 8 can be found in the specification at 29, lines 24-26.

Support for claims 9 and 10 can be found in the specification at page 31, line 24 through page 32, line 17.

Support for claim 11 can be found in originally filed claim 3 and in the specification at page 8, line 28 through page 9, line 11.

Support for claims 12 and 13 can be found in originally filed claim 5.

Support for claim 14 can be found in originally filed claims 1 and 5.

Applicants respectfully request that art considered during the prosecution of the parent application be made of record and considered in connection with the present application. Further, applicants respectfully request that all of the presently pending claims be examined within a single group.

The Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§ 1.16 and 1.17 which may be required by this paper, or to credit any overpayment, to Deposit Account No. 50-0815.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: September 25, 2000

By: Dianna L. DeVore
Dianna L. DeVore
Registration No. 42,484

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"Express Mail" Mailing Label No. EL 563 386 315 US

Atty Docket No. OLIG-017CON

Date of Deposit September 25, 2000

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Wilhelm Albert Palmen Jr.
Typed or Printed Name of Person Mailing Paper or Fee

Signature of Person Mailing Paper or Fee

CONTINUATION PATENT APPLICATION

"METHOD FOR DETECTING NUCLEIC ACID SEQUENCES"

Dianna L. DeVore
Registration No. 42,484
BOZICEVIC, FIELD & FRANCIS LLP
200 Middlefield Road, Suite 200
Menlo Park, CA 94025

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METHOD FOR DETECTING NUCLEIC ACID SEQUENCES

FIELD OF THE INVENTION

The field of this invention is arrays having associated oligonucleotides and uses thereof.

BACKGROUND OF THE INVENTION

Arrays of binding agents, such as oligonucleotides, have become an increasingly important tool in the biotechnology industry and related fields. These arrays, in which a plurality of binding agents are deposited onto a solid support surface in the form of an array or pattern, find use in a variety of applications, including drug screening, nucleic acid sequencing, mutation analysis, and the like. One important use of arrays is in the analysis of differential gene expression, where the expression of genes in different cells, normally a cell of interest and a control, is compared and any discrepancies in expression are identified. In such assays, the presence of discrepancies indicates a difference in the classes of genes expressed in the cells being compared.

In methods of differential gene expression, arrays find use by serving as a substrate with bound binding fragments such as oligonucleotides. Nucleic acid sequences are obtained from analogous cells, tissues or organs of a healthy and diseased organism, and hybridized to the immobilized set of binding fragments associated with the array. Differences between the resultant hybridization patterns are then detected and related to differences in gene expression in the two sources.

A variety of different array technologies have been developed in order to meet the growing need of the biotechnology industry. Despite the wide variety of array technologies currently in preparation or available on the market, there is a continued need to identify new array devices to meet the needs of specific applications. Of particular interest are arrays which

provide increased binding affinity, because these allow the use of shorter binding fragments and fewer bound fragments can be used to obtain the results currently available with conventional technology. Also of interest is the development of an array capable of providing high throughput analysis of differential gene expression, where the array itself is reusable. Such an array is needed for a number of reasons such as decreasing experimental variability, confirming results, and for decreasing costs of such analysis.

SUMMARY OF THE INVENTION

A method for detecting nucleic acid sequences in two or more collections of nucleic acid molecules, the method comprising:

(a) providing an array of modified polynucleotides bound to a solid surface, each said modified polynucleotide comprising a determinable nucleic acid;

(b) contacting the array of modified polynucleotides with:

(i) a first collection of labeled nucleic acid comprising a sequence substantially complementary to a nucleic acid of said array, and

(ii) at least a second collection of labeled nucleic acid comprising a sequence substantially complementary to a modified polynucleotide of said array;

wherein the first and second labels are distinguishable from each other; and

(c) detecting hybridization of the first and second labeled complementary nucleic acids to nucleic acids of said arrays;

wherein the modified oligonucleotides are characterized by a characteristic selected from the group consisting of (a) a binding affinity of at least about 1.25 times that of a corresponding, non-modified oligonucleotide, (b) a pH stability of at least one hour at 37 C at a pH in a range of about 0.5 to 10; and (c) a nuclease resistance of at least twice that of a naturally occurring oligonucleotide having the same sequence and number of bases.

These and other objects, advantages, and features of the invention will become apparent to those skilled in the art upon reading the details of the oligonucleotides and uses thereof as more fully described below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1-7 illustrate the chemical structure of exemplary modifications that result in acid stability.

Figures 8-9 illustrate the chemical structure of end-blocked, acid stable molecules used in the invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

It is to be understood that this invention is not limited to the particular methodology, support surfaces, materials and modifications described and as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

It must be noted that as used herein and in the appended claims, the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “an oligonucleotide” may include a plurality of oligonucleotide molecules and "an oligonucleotide" may encompass a plurality of oligonucleotides and equivalents thereof known to those skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices and materials similar or equivalent to those described

herein can be used in the practice or testing of the invention, the preferred methods, devices and materials are now described.

All publications mentioned are incorporated herein by reference for the purpose of describing and disclosing, for example, materials, constructs, and methodologies that are described in the publications which might be used in connection with the presently described invention. The publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

DEFINITIONS

The terms "nucleic acid" and "nucleic acid molecule" as used interchangeably herein, refer to a molecule comprised of nucleotides, i.e., ribonucleotides, deoxyribonucleotides, or both. The term includes monomers and polymers of ribonucleotides and deoxyribonucleotides, with the ribonucleotide and/or deoxyribonucleotides being connected together, in the case of the polymers, via 5' to 3' linkages. However, linkages may include any of the linkages known in the nucleic acid synthesis art including, for example, nucleic acids comprising 5' to 2' linkages. The nucleotides used in the nucleic acid molecule may be naturally occurring or may be synthetically produced analogues that are capable of forming base-pair relationships with naturally occurring base pairs. Examples of non-naturally occurring bases that are capable of forming base-pairing relationships include, but are not limited to, aza and deaza pyrimidine analogues, aza and deaza purine analogues, and other heterocyclic base analogues, wherein one or more of the carbon and nitrogen atoms of the purine and pyrimidine rings have been substituted by heteroatoms, *e.g.*, oxygen, sulfur, selenium, phosphorus, and the like.

The term "oligonucleotide" as used herein refers to a nucleic acid molecule comprising from about 2 to about 100 nucleotides, more preferably from 2 to 80 nucleotides, and even more preferably from about 4 to about 35 nucleotides.

The term "modified oligonucleotide" as used herein refer to oligonucleotides with one or more chemical modifications at the molecular level of the natural molecular structures of all or any of the bases, sugar moieties, internucleoside phosphate linkages, as well as molecules having added substituents, such as diamines, cholesterol or other lipophilic groups, or a combination of modifications at these sites. The internucleoside phosphate linkages can be phosphodiester, phosphotriester, phosphoramidate, siloxane, carbonate, carboxymethylester, acetamidate, carbamate, thioether, bridged phosphoramidate, bridged methylene phosphonate, phosphorothioate, methylphosphonate, phosphorodithioate, bridged phosphorothioate and/or sulfone internucleotide linkages, or 3'-3', 5'-2' or 5'-5' linkages, and combinations of such similar linkages (to produce mixed backbone modified oligonucleotides). The modifications can be internal (single or repeated) or at the end(s) of the oligonucleotide molecule and can include additions to the molecule of the internucleoside phosphate linkages, such as cholesteryl, diamine compounds with varying numbers of carbon residues between amino groups and terminal ribose, deoxyribose and phosphate modifications which cleave or cross-link to the opposite chains or to associated enzymes or other proteins. Electrophilic groups such as ribose-dialdehyde could covalently link with an epsilon amino group of the lysyl-residue of such a protein. A nucleophilic group such as *n*-ethylmaleimide tethered to an oligomer could covalently attach to the 5' end of an mRNA or to another electrophilic site. The term "modified oligonucleotides" also includes oligonucleotides comprising modifications to the sugar moieties such as 2'-substituted ribonucleotides, or deoxyribonucleotide monomers, any of which are connected together via 5' to 3' linkages. Modified oligonucleotides may also be comprised of PNA or morpholino modified backbones where target specificity of the sequence is maintained. A modified oligonucleotide of the invention (1) does not have the structure of a naturally

occurring oligonucleotide and (2) will hybridize to a natural oligonucleotide. Further, the modification preferably provides (3) higher binding affinity, (4) greater acid resistance, and (5) better stability against digestion with enzymes as compared to a natural oligonucleotide.

The term "oligonucleotide backbone" as used herein refers to the structure of the chemical moiety linking nucleotides in a molecule. The invention preferably comprises a backbone which is different from a naturally occurring backbone and is further characterized by holding bases in correct sequential order and (2) holding bases a correct distance between each other to allow a natural oligonucleotide to hybridize to it. This may include structures formed from any and all means of chemically linking nucleotides. A modified backbone as used herein includes modifications (relative to natural linkages) to the chemical linkage between nucleotides, as well as other modifications that may be used to enhance stability and affinity, such as modifications to the sugar structure. For example an a-anomer of deoxyribose may be used, where the base is inverted with respect to the natural b-anomer. In a preferred embodiment, the 2'-OH of the sugar group may be altered to 2'-O-alkyl or 2'-O-alkyl-n(O-alkyl), which provides resistance to degradation without comprising affinity.

The term “acidification” and “protonation/acidification” as used interchangeably herein refers to the process by which protons (or positive hydrogen ions) are added to proton acceptor sites on an oligonucleotide. The proton acceptor sites include the amine groups on the base structures of the oligonucleotide and the phosphate of the phosphodiester linkages. As the pH is decreased, the number of these acceptor sites which are protonated increases, resulting in a more highly protonated/acidified oligonucleotide.

The term "protonated/acidified oligonucleotide" refers to an oligonucleotide that, when dissolved in water at a concentration of approximately 16 A_{260} per ml, has a pH lower than physiological pH, i.e., lower than approximately pH 7. Modified oligonucleotides, nuclease-resistant oligonucleotides, and antisense oligonucleotides may all be encompassed by this definition. Generally, oligonucleotides are protonated/acidified by adding protons to the

reactive sites on an oligonucleotide via exposure of the oligonucleotide to an acidic environment, *e.g.*, exposure to an organic or mineral acid. Other modifications that will decrease the pH of the oligonucleotide can also be used and are intended to be encompassed by this term.

The term "end-blocked" as used herein refers to an oligonucleotide with a chemical modification at the molecular level that prevents the degradation of selected nucleotides, *e.g.*, by nuclease action. This chemical modification is positioned such that it protects the integral portion of the oligonucleotide, for example the coding region of an antisense oligonucleotide. An end block may be a 3' end block or a 5' end block. For example, a 3' end block may be at the 3'-most position of the molecule, or it may be internal to the 3' ends, provided it is 3' of the integral sequences of the oligonucleotide.

The term "substantially nuclease resistant" refers to oligonucleotides that are resistant to nuclease degradation, as compared to naturally occurring or unmodified oligonucleotides. Modified oligonucleotides of the invention are at least 1.25 times more resistant to nuclease degradation than their unmodified counterpart, more preferably at least 2 times more resistant, even more preferably at least 5 times more resistant, and most preferably at least 10 times more resistant than their unmodified counterpart. Such substantially nuclease resistant oligonucleotides include, but are not limited to, oligonucleotides with modified backbones such as phosphorothioates, methylphosphonates, ethylphosphotriesters, 2'-O-methylphosphorothioates, 2'-O-methyl-p-ethoxy ribonucleotides, 2'-O-alkyls, 2'-O-alkyl-n(O-alkyl), 2'-fluoros, 2'-deoxy-erythropentofuranosyls, 2'-O-methyl ribonucleosides, methyl carbamates, methyl carbonates, inverted bases (*e.g.*, inverted T's), or chimeric versions of these backbones.

The term "substantially acid resistant" as used herein refers to oligonucleotides that are resistant to acid degradation as compared to unmodified oligonucleotides. Typically, the relative acid resistance of an oligonucleotide will be measured by comparing the percent

degradation of a resistant oligonucleotide with the percent degradation of its unmodified counterpart (i.e., a corresponding oligonucleotide with "normal" backbone, bases, and phosphodiester linkages). An oligonucleotide that is acid resistant is preferably at least 1.5 times more resistant to acid degradation, at least 2 times more resistant, even more preferably at least 5 times more resistant, and most preferably at least 10 times more resistant than their unmodified counterpart.

The term "alkyl" as used herein refers to a branched or unbranched saturated hyrdrocarbon chain containing 1-6 carbon atoms, such as methyl, ethyl, propyl, tert-butyl, n-hexyl and the like.

The term "array type" refers to the type of gene represented on the array by the associated test oligonucleotides, where the type of gene that is represented on the array is dependent on the intended purpose of the array, *e.g.*, to monitor expression of key human genes, to monitor expression of known oncogenes, etc., i.e., the use for which the array is designed. As such, all of the test oligonucleotides on a given array correspond to the same type or category or group of genes. Genes are considered to be of the same type if they share some common linking characteristics, such as: species of origin, *e.g.*,, human, mouse, rat, etc.; tissue or cell type of origin, *e.g.*, muscle, neural, dermal, organ, etc.; disease state, *e.g.*, cancer; functions, *e.g.*, protein kinases, tumor supressors and the like; participation in the same normal biological process, *e.g.*, apoptosis, signal transduction, cell cycle regulation, proliferation, differentiation etc.; and the like. For example, one array type is a "cancer array" in which each of the "unique" associated test oligonucleotides correspond to a gene associated with a cancer disease state. Likewise, a "human array" may be an array of test oligonucleotides corresponding to unique tightly regulated human genes. Similarly, an "apoptosis array" may be an array type in which the associated test oligonucleotides correspond to unique genes associated with apoptosis.

The terms "associated oligonucleotide" and "substrate oligonucleotide" refer to the oligonucleotide composition that makes up each of the samples associated to the array. Thus, the term "associated oligonucleotide" includes oligonucleotide compositions of unique sequences and/or control or calibrating sequences (*e.g.*, oligonucleotides corresponding to housekeeping genes). The oligonucleotide compositions are preferably comprised of single stranded oligonucleotides, where all of the oligonucleotides in a sample composition may be identical to each other. Alternatively, there may be oligonucleotides of two or more sequences in each composition, for example two different oligonucleotides that are separate but complementary to each other.

THE INVENTION IN GENERAL

Oligonucleotides with modified backbone structures, such as oligonucleotides with 2'-O-alkyl and 2'-O-alkyl-n(O-alkyl) sugar moieties and/or 3' linkage modifications, are provided. Modified oligonucleotides of the invention also may be acid resistant and/or exonuclease resistant to further decrease the sensitivity of the oligonucleotide molecule. Preferably, the exonuclease resistant block is added to the 3' or the 5' end of the oligonucleotide depending on the attachment of the oligonucleotide to the substrate. The resulting modified oligonucleotides of the invention bind tightly to their RNA or DNA targets.

Modified oligonucleotides of the invention preferably have an increased binding affinity over their non-modified counterparts. This binding affinity can be determined using T_m assays such as those described in L.L.Cummins et al, Nucleic Acids Research 23:2019-2024 (1995). Typically, the T_m of an oligonucleotide will increase approximately 1 C for each 2'-O-methyl substitution in a molecule, and the T_m increases even more for 2'-O-propyl and 2'-F substitutions. Thus, in one embodiment, the T_m of the substituted oligonucleotide is 2-15 C, and even more preferably 8-10 C higher than the corresponding non-modified oligonucleotide.

Acid stable associated oligonucleotides of the invention are stable when exposed to a pH of 1-2, while their binding partners are not. This allows an array having associated protonated oligonucleotides to be exposed to a first sample, treated with an acidic solution applied in any of several possible protocols to free the array from the first binding partner, and reused with a second sample. Direct comparison of two different samples of binding partners using a single array has the advantage of limiting potential experimental variation present when comparing multiple arrays. Performing the experiment with the same sample on the same array allows a confirmation of the results obtained in the first instance, thus effectively confirming results without having variation in the array composition.

Similarly, associated end-blocked oligonucleotides display a resistance to nucleases, allowing the arrays to be exposed to DNA nucleases to free the array from a sample of binding partners. An array of the invention having nuclease resistant associated oligonucleotides can be treated with an appropriate nuclease and reused with a different or the same sample.

The arrays of the present invention encompass associated oligonucleotides chemically modified to be acid stable from a pH of 0.01 to 7.0, and more preferably acid stable in a pH of 1.0 to 4.0, allowing such molecules to retain their structural integrities in acidic environments. Although any 2' modified oligonucleotide may be used in the present invention, in a preferred embodiment the oligonucleotides of the invention are 2'-O-alkyl and 2'-O-alkyl-n(O-alkyl) oligonucleotides which, unlike phosphodiester or phosphorothioate DNA or RNA, exhibit significant acid resistance in solutions with pH as low as 0-1 even at 37 C. Acid stability of this first component coupled with the introduction of 3' or 3' and 5' acid stable, exonuclease resistant ends, confers several unique properties on 2'-O-alkyl and 2'-O-alkyl-n(O-alkyl) oligonucleotides. These low toxicity, highly specific, acid stable, end-blocked 2'-O-alkyl and 2'-O-alkyl-n(O-alkyl) oligonucleotides represent a novel and improved oligonucleotide structure for use in array technologies.

Typically, the relative nuclease resistance of a oligonucleotide can be measured by comparing the percent digestion of a resistant oligonucleotide with the percent digestion of its unmodified counterpart (i.e., a corresponding oligonucleotide with "normal" backbone, bases, and phosphodiester linkage). Percent degradation may be determined by using analytical HPLC to assess the loss of full length oligonucleotides, or by any other suitable methods (*e.g.*, by visualizing the products on a sequencing gel using staining, autoradiography, fluorescence, etc., or measuring a shift in optical density). Degradation is generally measured as a function of time.

Comparison between unmodified and modified oligonucleotides can be made by ratioing the percentage of intact modified oligonucleotide to the percentage of intact unmodified oligonucleotide. For example, if, after 15 minutes of exposure to a nuclease, 25% (i.e., 75% degraded) of an unmodified oligonucleotide is intact, and 50% (i.e., 50% degraded) of a modified oligonucleotide is intact, the modified oligonucleotide is said to be 2 times (50% divided by 25%) more resistant to nuclease degradation than is the unmodified oligonucleotide. Generally, a substantially nuclease resistant oligonucleotide will be at least about 1.25 times more resistant to nuclease degradation than an unmodified oligonucleotide with a corresponding sequence, typically at least about 1.5 times more resistant, preferably about 1.75 times more resistant, and more preferably at least about 10 times more resistant after 15 minutes of nuclease exposure.

Percent acid degradation may be determined by using analytical HPLC to assess the loss of full length oligonucleotides, or by any other suitable methods (*e.g.*, by visualizing the products on a sequencing gel using staining, autoradiography, fluorescence, etc., or measuring a shift in optical density). Degradation is generally measured as a function of time.

Comparison between unmodified and modified oligonucleotides can be made by ratioing the percentage of intact modified oligonucleotide to the percentage of intact unmodified oligonucleotide. For example, if, after 30 minutes of exposure to a low pH environment, 25%

(i.e., 75% degraded) of an unmodified oligonucleotide is intact, and 50% (i.e., 50% degraded) of a modified oligonucleotide is intact, the modified oligonucleotide is said to be 2 times (50% divided by 25%) more resistant to nuclease degradation than is the unmodified oligonucleotide. Generally, substantially "acid resistant" oligonucleotides will be at least about 1.25 times more resistant to acid degradation than an unmodified oligonucleotide with a corresponding sequence, typically at least about 1.5 times more resistant, preferably about 1.75 more resistant, more preferably at least 5 times more resistant and even more preferably at least about 10 times more resistant after 30 minutes of exposure at 37 C to a pH of about 1.5 to about 4.5.

Acidification of oligonucleotides is the process by which protons (or positive hydrogen ions) are added to the reactive sites on an oligonucleotide. As the number of reactive sites that are protonated increases, the pH is decreased, and the bacterial inhibiting activity of the oligonucleotide is increased. Accordingly, the oligonucleotides of the invention are protonated/acidified to give a pH when dissolved in water of less than pH 7 to about pH 1, or in preferred embodiments, pH 6 to about 1 or pH 5 to about 1. In other preferred embodiments, the dissolved oligonucleotides have a pH from pH 4.5 to about 1 or, in a preferred embodiment, a pH of 4.0 to about 1, or, in a more preferred embodiment, a pH of 3.0 to about 1, or, in another more preferred embodiment, a pH of 2.0 to about 1.

In a preferred embodiment, the end-blocked oligonucleotides of the compositions are further acidified/protonated and methods of the invention are substantially nuclease resistant, substantially acid resistant, and preferably, both substantially nuclease resistant and substantially acid resistant. This embodiment includes oligonucleotides completely or partially derivatized by one or more linkages from the group comprised of phosphorothioate linkages, 2'-O-methyl-phosphodiesters, 2'-O-alkyl, 2'-O-ethyl, 2'-O-propyl, 2'-O-butyl, 2'-O-alkyl-n(O-alkyl), 2'-methoxyethoxy, 2'-fluoro, 2'-deoxy-erythropentofuranosyl, 3'-O-methyl, p-isopropyl oligonucleotides, phosphodiester, 2'-O$(CH_2CH_2O)_xCH_3$, butyne, phosphotriester, phosphoramidate, propargyl, siloxane, carbonate, carboxymethylester, methoxyethoxy,

acetamidate, carbamate, thioether, bridged phosphoramidate, bridged methylene phosphonate, methylphosphonate, phosphorodithioate, bridged phosphorothioate and/or sulfone internucleotide linkages, or 3'-3' or 5'-5' or 5'-2' linkages, and combinations of such similar linkages (to produce mixed backbone modified oligonucleotides), and any other backbone modifications.

Exemplary modifications that result in acid stability can be seen in Figures 1-6. End-blocked acid stable molecules are illustrated in Figures 7-8.

This embodiment also includes other modifications that render the oligonucleotides substantially resistant to nuclease activity. Methods of rendering an oligonucleotide nuclease resistant include, but are not limited to, covalently modifying the purine or pyrimidine bases that comprise the oligonucleotide. For example, bases may be methylated, hydroxymethylated, or otherwise substituted (*e.g.*, glycosylated) such that the oligonucleotides comprising the modified bases are rendered substantially nuclease resistant.

In a preferred embodiment, the oligonucleotide will have a backbone substantially resistant to acid degradation, exonuclease digestion, and endonuclease digestion. In the most preferred embodiment an oligonucleotide is uniformly modified with 2'-O-alkyl or 2'-O-alkyl-n(O-alkyl) groups, i.e., every base of the oligonucleotide is a 2'-O-alkyl or 2'-O-alkyl-n(O-alkyl) modified base.

In another embodiment, the oligonucleotides of the current invention are used for diagnostic purposes. For example, oligonucleotides of the current invention may be used to detect complementary oligonucleotides by contacting an oligonucleotide of the invention with an oligonucleotide sample under conditions that allow for the hybridization of the oligonucleotide of the invention to any complementary oligonucleotide present in the sample, and detecting such hybridization.

Oligonucleotides with a range of nuclease-resistant backbones were evaluated. As a result, a preferred embodiment of the present invention is an end-blocked oligonucleotide with

the chemical backbone structure of 5'-butanol-2'-O-alkyl RNA-butanol-3' or 2'-O-alkyl-O-alkyl. A particularly preferred embodiment of the present invention is a protonated/acidified oligonucleotide with the chemical backbone structure of 5'-butanol-2'-O-methyl RNA-butanol-3', 5'-butanol-2'-O-alkyl-O-alkyl RNA-butanol-3' or 2'-O-alkyl-O-alkyl RNA that has a pH of 3 to 1 when dissolved in water. The end-blocking group on one end of the oligonucleotide may not be needed, depending on the manner of association with the substrate, as will be apparent to one skilled in the art upon reading the present disclosure. Exemplary modifications for use on the present array can be found in U.S. Serial No. 09/223,498, and U.S. Serial No. 09/356,069, which are incorporated herein by reference in their entirety.

ARRAY CONSTRUCTION AND USES

The arrays of the subject invention have a plurality of associated modified oligonucleotides stably associated with a surface of a solid support, *e.g.*, covalently attached to the surface with or without a linker molecule. Each associated sample on the array comprises a modified oligonucleotide composition, of known identity, usually of known sequence, as described in greater detail below. Any conceivable substrate may be employed in the invention.

In the arrays of the invention, the modified oligonucleotide compositions are stably associated with the surface of a solid support, where the support may be a flexible or rigid solid support. By "stably associated" is meant that the sample of associated modified oligonucleotides maintain their position relative to the solid support under hybridization and washing conditions. As such, the samples can be non-covalently or covalently stably associated with the support surface. Examples of non-covalent association include non-specific adsorption, binding based on electrostatic interactions (*e.g.*, ion, ion pair interactions), hydrophobic interactions, hydrogen bonding interactions, specific binding through a specific binding pair member covalently attached to the support surface, and the like. Examples of covalent binding include covalent bonds formed between the oligonucleotides and a functional group present on the surface of the

rigid support, *e.g.*, -OH, where the functional group may be naturally occurring or present as a member of an introduced linking group, as described in greater detail below.

As mentioned above, the array is present on either a flexible or rigid substrate. A flexible substrate is capable of being bent, folded or similarly manipulated without breakage. Examples of solid materials which are flexible solid supports with respect to the present invention include membranes, *e.g.*, nylon, flexible plastic films, and the like. By "rigid" is meant that the support is solid and does not readily bend, i.e., the support is not flexible. As such, the rigid substrates of the subject arrays are sufficient to provide physical support and structure to the associated oligonucleotides present thereon under the assay conditions in which the array is employed, particularly under high throughput handling conditions. Furthermore, when the rigid supports of the subject invention are bent, they are prone to breakage.

The substrate may be biological, nonbiological, organic, inorganic, or a combination of any of these, existing as particles, strands, precipitates, gels, sheets, tubing, spheres, containers, capillaries, pads, slices, films, plates, slides, etc. The substrate may have any convenient shape, such as a disc, square, sphere, circle, etc.

The substrate is preferably flat but may take on a variety of alternative surface configurations. For example, the substrate may contain raised or depressed regions on which the synthesis takes place. The substrate and its surface preferably form a rigid support on which to carry out the reactions described herein. The substrate and its surface is also chosen to provide appropriate light-absorbing characteristics. For instance, the substrate may be a polymerized Langmuir Blodgett film, functionalized glass, Si, Ge, GaAs, GaP, SiO_2, SIN_4, modified silicon, or any one of a wide variety of gels or polymers such as (poly)tetrafluoroethylene, (poly)vinylidenedifluoride, polystyrene, polycarbonate, or combinations thereof.

Other substrate materials will be readily apparent to those of skill in the art upon review of this disclosure. In a preferred embodiment the substrate is flat glass or single-crystal silicon

with surface relief features of less than 10 angstroms. According to some embodiments, the surface of the substrate is etched using well known techniques to provide for desired surface features. For example, by way of the formation of trenches, v-grooves, mesa structures, or the like, the synthesis regions may be more closely placed within the focus point of impinging light, be provided with reflective "mirror" structures for maximization of light collection from fluorescent sources, or the like.

Surfaces on the solid substrate will usually, though not always, be composed of the same material as the substrate. Thus, the surface may be composed of any of a wide variety of materials, for example, polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, membranes, or any of the above-listed substrate materials. In some embodiments the surface may provide for the use of caged binding members which are attached firmly to the surface of the substrate. Preferably, the surface will contain reactive groups, which could be carboxyl, amino, hydroxyl, or the like. Most preferably, the surface will be optically transparent and will have surface Si--OH functionalities, such as are found on silica surfaces.

The surface of the substrate is preferably provided with a layer of linker molecules, although it will be understood that the linker molecules are not required elements of the invention. The linker molecules are preferably of sufficient length to permit modified oligonucleotides of the invention and on a substrate to hybridize to natural oligonucleotides and to interact freely with molecules exposed to the substrate. The linker molecules should be 6-50 atoms long to provide sufficient exposure. The linker molecules may be, for example, aryl acetylene, ethylene glycol oligomers containing 2-10 monomer units, diamines, diacids, amino acids, or combinations thereof. Other linker molecules which can bind to modified oligonucleotides of the invention may be used in light of this disclosure.

According to another alternative embodiment, linker molecules are also provided with a photocleavable group at an intermediate position. The photocleavable group is preferably

cleavable at a wavelength different from the protective group. This enables removal of the various polymers following completion of the synthesis by way of exposure to the different wavelengths of light.

The linker molecules can be attached to the substrate via carbon-carbon bonds using, for example, (poly)trifluorochloroethylene surfaces, or preferably, by siloxane bonds (using, for example, glass or silicon oxide surfaces). Siloxane bonds with the surface of the substrate may be formed in one embodiment via reactions of linker molecules bearing trichlorosilyl groups. The linker molecules may optionally be attached in an ordered array, i.e., as parts of the head groups in a polymerized Langmuir Blodgett film. In alternative embodiments, the linker molecules are adsorbed to the surface of the substrate.

In one embodiment of the present invention, the linker molecules and modified nucleotides used herein are provided with a functional group to which is bound a protective group. Preferably, the protective group is on the distal or terminal end of the linker molecule opposite the substrate. The protective group may be either a negative protective group (i.e., the protective group renders the linker molecules less reactive with a monomer upon exposure) or a positive protective group (i.e., the protective group renders the linker molecules more reactive with a monomer upon exposure). In the case of negative protective groups an additional step of reactivation will be required. In some embodiments, this will be done by heating. The protective group on the linker molecules may be selected from a wide variety of positive light-reactive groups preferably including nitro aromatic compounds such as o-nitrobenzyl derivatives or benzylsulfonyl. In a preferred embodiment, 6-nitroveratryloxycarbonyl (NVOC), 2-nitrobenzyloxycarbonyl (NBOC) or , -dimethyl-dimethoxybenzyloxycarbonyl (DDZ) is used. Photoremovable protective groups are described in, for example, Patchornik, J. Am. Chem. Soc. (1970) 92:6333 and Amit et al., J. Org. Chem. (1974) 39:192, both of which are incorporated herein by reference.

The substrate, the area of synthesis, and the area for synthesis of each individual

oligonucleotide group could be of any size or shape. For example, squares, ellipsoids, rectangles, triangles, circles, or portions thereof, along with irregular geometric shapes, may be utilized. Duplicate synthesis regions may also be applied to a single substrate for purposes of redundancy.

The regions on the substrate can have a surface area of between about 1 cm^2 and 10^{-10} cm^2. Preferably, the regions have areas of less than about 10^{-1} to 10^{-7} cm^2, more preferably less than 10^{-3} to 10^{-6} cm^2, and even more preferably less than 10^{-5} cm^2.

A single substrate supports more than about 10 different oligonucleotide sequences and preferably more than about 100 different oligonucleotide sequences, although in some embodiments more than about 10^3, 10^4, 10^5, 10^6, 10^7, or 10^8 different sequences are provided on a substrate. Of course, within a region of the substrate in which a modified oligonucleotide is synthesized, it is preferred that the modified nucleotides be substantially pure. In preferred embodiments, regions of the substrate contain oligonucleotides which are at least about 50%, preferably 80%, more preferably 90%, and even more preferably, 95% pure. Several sequences can be intentionally provided within a single region so as to provide an initial screening for biological activity, after which materials within regions exhibiting significant binding are further evaluated. In a preferred embodiment, each region will contain a substantially pure modified oligonucleotide with a single sequence.

The method and apparatus includes use of selected printing techniques in distributing materials such as barrier materials, deprotection agents, base groups, nucleosides, nucleotides, nucleotide analogs, amino acids, imino acids, carrier materials, and the like to selected regions of a substrate. Each of the printing techniques may be used in some embodiments with, for example, standard DMT-based chemistry for synthesis of oligonucleotides, and in particular selected deprotecting agents in vapor form.

The substrates of the arrays of the invention comprise at least one surface on which the pattern of associated oligonucleotides is present, where the surface may be smooth or substantially planar, or have irregularities, such as depressions or elevations. The surface on which the pattern

of associated oligonucleotides is present may be modified with one or more different layers of compounds that serve to modify the properties of the surface in a desirable manner. Such modification layers, when present, will generally range in thickness from a monomolecular thickness to about 1 mm, usually from a monomolecular thickness to about 0.1 mm and more usually from a monomolecular thickness to about 0.001 mm. Modification layers of interest include: inorganic and organic layers such as metals, metal oxides, polymers, small organic molecules and the like.

The amount of modified oligonucleotide present in each sample will be sufficient to provide for adequate hybridization and detection of test nucleic acids during the assay in which the array is employed. Generally, the amount of oligonucleotide in each sample will be at least about 0.1 ng, usually at least about 0.5 ng and more usually at least about 1 ng, where the amount may be as high as 1000 ng or higher, but will usually not exceed about 20 ng and more usually will not exceed about 10 ng. The copy number of each oligonucleotide in a sample will be sufficient to provide enough hybridization sites to yield a detectable signal, and will generally range from about 0.01 fmol to 50 fmol, usually from about 0.05 fmol to 20 fmol and more usually from about 0.1 fmol to 5 fmol. Where the sample has an overall circular dimension, the diameter of the sample will generally range from about 10 to 5,000 m, usually from about 20 to 2,000 m and more usually from about 50 to 1000 m.

Control samples may be present on the array including samples comprising oligonucleotides corresponding to genomic DNA, housekeeping genes, negative and positive control genes, and the like. These latter types of samples comprise oligonucleotide compositions that are not "unique" as that term is defined and used herein, i.e., they are "common." In other words, they are calibrating or control genes whose function is not to tell whether a particular "key" gene of interest is expressed, but rather to provide other useful information, such as background or basal level of expression, and the like. The percentage of samples which are made of unique oligonucleotides that correspond to the same type of gene is generally at least about 30%, and usually at least about 60%

and more usually at least about 80%. Preferably, the arrays of the present invention will be of a specific type, where representative array types include: human arrays, mouse arrays, cancer arrays, apoptosis arrays, human stress arrays, oncogene and tumor suppressor arrays, cell-cell interaction arrays, cytokine and cytokine receptor arrays, rat arrays, blood arrays, mouse stress arrays, neuroarrays, and the like.

With respect to the oligonucleotide compositions that correspond to a particular type or kind of gene, type or kind can refer to a plurality of different characterizing features, where such features include: species specific genes, where specific species of interest include eukaryotic species, such as mice, rats, rabbits, pigs, primates, humans, etc.; function specific genes, where such genes include oncogenes, apoptosis genes, cytokines, receptors, protein kinases, etc.; genes specific for or involved in a particular biological process, such as apoptosis, differentiation, cell cycle regulation, cancer, aging, proliferation, etc.; location specific genes, where locations include organs, such as heart, liver, prostate, lung etc.; tissue, such as nerve, muscle, connective, etc.; cellular, such as axonal, lymphocytic, etc.; or subcellular locations, *e.g.*, nucleus, endoplasmic reticulum, Golgi complex, endosome, lyosome, peroxisome, mitochondria, cytoplasm, cytoskeleton, plasma membrane, extracellular space; specific genes that change expression level over time, *e.g.*, genes that are expressed at different levels during the progression of a disease condition, such as prostate genes which are induced or repressed during the progression of prostate cancer.

The average length of the associated modified oligonucleotides on the array is chosen to be of sufficient length to provide a strong and reproducible signal, as well as tight and robust hybridization. As such, the average length of the oligonucleotides of the array will typically range from about 4 to 80 nucleotides, and more preferably about 10 to about 35 nucleotides.

As mentioned above, the arrays of the present invention typically comprise one or more additional associated oligonucleotide sample which does not correspond to the array type, i.e., the type or kind of gene represented on the array. In other words, the array may comprise one or more

samples that are made of non "unique"oligonucleotides, *e.g.*, oligonucleotides corresponding to commonly expressed genes. For example, samples comprising oligonucleotides that bind to plasmid and bacteriophage oligonucleotides, oligonucleotides which bind to genes from the same or another species which are not expressed and do not cross-hybridize with the test nucleic acid, and the like, may be present and serve as negative controls. In addition, samples comprising housekeeping genes and other control genes from the same or another species may be present, which samples serve in the normalization of mRNA abundance and standardization of hybridization signal intensity in the sample assayed with the array.

Patents and patent applications describing arrays of oligonucleotides and methods for their fabrication include: 5,242,974; 5,384,261; 5,405,783; 5,412,087; 5,424,186; 5,429,807; 5,436,327; 5,445,934; 5,472,672; 5,527,681; 5,529,756; 5,545,531; 5,554,501; 5,556,752; 5,561,071; 5,599,895; 5,624,711; 5,639,603; 5,658,734; 5,700,637; 5,744,305; 5,837,832; 5,843,655; 5,861,242; 5,874,974; 5,885,837; WO 93/17126; WO 95/11995; WO 95/35505; EP 742 287; and EP 799 897. Patents and patent applications describing methods of using arrays in various applications include: 5,143,854; 5,288,644; 5,324,633; 5,432,049; 5,470,710; 5,492,806; 5,503,980; 5,510,270; 5,525,464; 5,547,839; 5,580,732; 5,661,028; 5,848,659; 5,874,219; WO 95/21265; WO 96/31622; WO 97/10365; WO 97/27317; EP 373 203; and EP 785 280. References that disclose the synthesis of arrays and reagents for use with arrays include: Matteucci M. D. and Caruthers M. H. J. Am. Chem. Soc. 1981, 103, 3185-3191; Beaucage S. L. and Caruthers M. H. Tetrahedron Letters, Vol 22, No.20, pp 1859-1862, 1981; Adams S. P. et al, J.Am. Chem. Soc. 1983, 105, 661-663; Sproat D. S. and Brown D. M. Nucleic Acids Research, Vol 13, No.8, 1985, 2979-2987; Crea R. and Horn T., Nucleic Acids Research, 8, No 10, 1980, 2331-48; Andrus A. et al., Tetrahedron Letters, Vol 29, No. 8, pp 861-4, 1988; Applied Biosystems User Bulletin, Issue No. 43, Oct. 1, 1987, "Methyl phosphonamidite reagents and the synthesis and purification of methyl phosphonate analogs of DNA"; Miller P. S. et al., Nucleic Acids Research, 11, pages 6225-6242, 1983; Each of these is incorporated herein by reference as exemplary methods of

construction and use of arrays of the present invention. The methods of these publications can be readily modified to produce the arrays of the invention with the modified oligonucleotides of the invention on their surface.

One preferred method of the invention uses ink jet printing technology to place modified nucleotides of the invention in their correct spot on a substrate. In place of ink four different solutions are used with each containing a substantially pure solution of one of the four bases A, T, G or C. The printer technology then places the correct base on the correct spot and builds the desired oligonucleotide of the invention in place. By correctly programming the printer it is possible to "print" the desired pattern of modified oligonucleotides on the substrate.

ASSOCIATED OLIGONUCLEOTIDE COMPOSITIONS OF THE ARRAYS

Each associated oligonucleotide composition of the pattern present on the surface of the substrate is preferably made up of a set of unique oligonucleotides, and preferably a unique oligonucleotide composition. By "unique composition" is meant a collection or population of single stranded oligonucleotides capable of participating in a hybridization event under appropriate hybridization conditions, where each of the individual oligonucleotides may be the same -- have the same nucleotide sequence -- or different sequences, for example the oligonucleotide composition may consist of two different oligonucleotides that are complementary to each other (i.e., the two different oligonucleotides are complementary but physically separated so as to be single stranded, i.e., not hybridized to each other). In many embodiments, the oligonucleotide compositions will comprise two complementary, single stranded oligonucleotides.

In those compositions having unique oligonucleotides, the sequence of the oligonucleotides are chosen in view of the type and the intended use of the array on which they are present. The unique oligonucleotides are preferably chosen so that each distinct unique oligonucleotide does not cross-hybridize with any other distinct unique oligonucleotide on the array, i.e., the oligonucleotide will not cross-hybridize to any other oligonucleotide compositions that corresponds to a different

gene falling within the broad category or type of genes represented on the array under appropriate conditions. As such, the nucleotide sequence of each unique oligonucleotide of a composition will have less than 90% homology, usually less than 85 % homology, and more usually less than 80% homology with any other different associated oligonucleotide composition of the array, where homology is determined by sequence analysis comparison using the FASTA program using default settings. The sequence of unique associated oligonucleotides in the compositions are not conserved sequences found in a number of different genes (at least two), where a conserved sequence is defined as a stretch of from about 4 to about 80 nucleotides which have at least about 90% sequence identity, where sequence identity is measured as above. The associated oligonucleotide will generally have a length of from about 4 to about 80 nucleotides, usually from 10 to about 40 nucleotides, and more usually 15-35 nucleotides. The length of the nucleic acid can be chosen to best provide binding to the test sequence.

Although in a preferred embodiment the associated oligonucleotide composition will not cross-hybridize with any other associated oligonucleotides on the array under standard hybridization conditions, associated oligonucleotides and hybridization conditions can be altered to allow binding to multiple associated oligonucleotide compositions. For example, in determining the relatedness of a sample to oligonucleotides representing different members of a class of proteins, the oligonucleotide sequences may be more similar and/or less stringent hybridization conditions may be used.

PREPARATION AND USE OF ARRAYS OF THE INVENTION

Polynucleotide arrays provide a high throughput technique that can assay a large number of polynucleotide sequences in a sample. By "array" is meant an article of manufacture that has at least a substrate with at least two distinct, associated modified oligonucleotides on one of its surfaces, where the number of distinct oligonucleotides can be considerably higher, typically being at least 10 nucleotides, usually at least 20 nucleotides, and often at least 25 nucleotides. A variety

of different array formats have been developed and are known to those of skill in the art. The arrays of the subject invention find use in a variety of applications, including gene expression analysis, drug screening, mutation analysis and the like.

Arrays can be created by spotting polynucleotide probes onto a substrate (*e.g.*, glass, nitrocelllose, etc.) in a two-dimensional matrix or array having bound probes. The probes can be bound to the substrate by either covalent bonds or by non-specific interactions, such as hydrophobic interactions. Samples of polynucleotides can be detectably labeled (*e.g.*, using radioactive or fluorescent labels) and then hybridized to the probes. Double stranded polynucleotides, comprising the labeled sample polynucleotides bound to probe polynucleotides, can be detected once the unbound portion of the sample is washed away. Techniques for constructing arrays and methods of using these arrays are described in EP 799 897; WO 97/29212; WO 97/27317; EP 785 280; WO 97/02357; USPN 5,593,839; USPN 5,578,832; EP 728 520; USPN 5,599,695; EP 721 016; USPN 5,556,752; WO 95/22058; and USPN 5,631,734.

Arrays can be used to, for example, examine differential expression of genes and can be used to determine gene function. For example, arrays can be used to detect differential expression of a polynucleotide between a test cell and control cell (*e.g.*, cancer cells and normal cells). For example, high expression of a particular message in a cancer cell, which is not observed in a corresponding normal cell, can indicate a cancer specific gene product. Exemplary uses of arrays are further described in, for example, Pappalarado *et al.*, *Sem. Radiation Oncol.* 8:217 (1998), and Ramsay *Nature Biotechnol.* 16:40 (1998).

The oligonucleotide on the array will usually be at least about 4-80 nucleotides, more preferably 10-35 nucleotides, and usually at least 12 nucleotides in length. Reference arrays can be produced according to any suitable methods known in the art. For example, methods of producing large arrays of oligonucleotides are described in U.S. 5,134,854, and U.S. 5,445,934 using light-directed synthesis techniques. Using a computer controlled system, a heterogeneous array of monomers is converted, through simultaneous coupling at a number of reaction sites, into

a heterogeneous array of polymers. Alternatively, microarrays are generated by deposition of pre-synthesized oligonucleotides onto a solid substrate, for example as described in PCT published application no. WO 95/35505.

Methods for analyzing the data collected from hybridization to arrays are well known in the art. For example, where detection of hybridization involves a fluorescent label, data analysis can include the steps of determining fluorescent intensity as a function of substrate position from the data collected, removing outliers, i.e., data deviating from a predetermined statistical distribution, and calculating the relative binding affinity of the test nucleic acids from the remaining data. The resulting data can be displayed as an image with the intensity in each region varying according to the binding affinity between associated oligonucleotides and the test nucleic acids.

Oligonucleotides having a sequence unique to that gene are preferab;y used in the present invention. Different methods may be employed to choose the specific region of the gene to be targeted. A rational design approach may also be employed to choose the optimal oligonucleotide sequence for the hybridization array. Preferably, the region of the gene that is selected is chosen based on the following criteria. First, the sequence that is chosen should yield a oligonucleotide composition that preferably does not cross-hybridize with any other oligonucleotide composition present on the array. Second, the sequence should be chosen such that the oligonucleotide composition has a low probability of cross-hybridizing with an oligonucleotide having a nucleotide sequence found in any other gene, whether or not the gene is to be represented on the array from the same species of origin, *e.g.*, for a human array, the sequence will not be present in any other human genes. As such, sequences that are avoided include those found in: highly expressed gene products, structural RNAs, repeated sequences found in the sample to be tested with the array and sequences found in vectors. A further consideration is to select sequences that provide for minimal or no secondary structure, structure which allows for optimal hybridization but low non-specific binding, equal or similar thermal stabilities, and optimal hybridization characteristics.

Prepared modified oligonucleotide compositions may be associated on the support using any convenient methodology. The arrays may also be produced using *in situ* synthesis of modified

oligonucleotides on the array directly using techniques for such synthesis available in the art. Such synthesis protocols include manual techniques, *e.g.*, by micro pipette, ink jet, pins, etc., as well as automated protocols. . As mentioned above, the oligonucleotide compositions that are associated to the array surface are made up of single stranded oligonucleotides, where all the oligonucleotides may be identical to each other or a population of complementary oligonucleotides may be present in each sample.

OLIGONUCLEOTIDE SYNTHESIS

Oligonucleotides can be synthesized on commercially purchased DNA synthesizers from $<$ 1uM to $>$ 1mM scales using standard phosphoramidite chemistry and methods that are well known in the art, such as, for example, those disclosed in Stec *et al.*, *J. Am. Chem. Soc.* 106:6077-6089 (1984), Stec *et al.*, *J. Org. Chem.* 50(20):3908-3913 (1985), Stec *et al.*, *J. Chromatog.* 326:263-280 (1985), LaPlanche *et al.*, *Nuc. Acid. Res.* 14(22):9081-9093 (1986), and Fasman, *Practical Handbook of Biochemistry and Molecular Biology*, 1989, CRC Press, Boca Raton, FL, herein incorporated by reference.

Oligonucleotides can be deprotected following phosphoramidite manufacturer's protocols. Unpurified oligonucleotides may be dried down under vacuum or precipitated and then dried. Sodium salts of oligonucleotides can be prepared using the commercially available DNA-Mate (Barkosigan Inc.) reagents or conventional techniques such as a commercially available exchange resin, *e.g.*, Dowex, or by addition of sodium salts followed by precipitation, diafiltration, or gel filtration, etc.

Oligonucleotides to be purified can be chromatographed on commercially available reverse phase or ion exchange media, *e.g.*, Waters Protein Pak, Pharmacia's Source Q, etc. Peak fractions can be combined and the samples desalted and concentrated by means of reverse phase chromatography on poly(styrene-divinylbenzene) based columns like Hamilton's PRP, or Polymer Labs PLRP.

Alternatively, ethanol precipitation, diafiltration, or gel filtration may be used followed by lyophilization or solvent evaporation under vacuum in commercially available instrumentation such as Savant's Speed Vac. Optionally, small amounts of the oligonucleotides may be electrophoretically purified using polyacrylamide gels.

Lyophilized or dried-down preparations of oligonucleotides can be dissolved in pyrogen-free, sterile, physiological saline (i.e., 0.85% saline), sterile Sigma water, and filtered through a 0.45 micron Gelman filter (or a sterile 0.2 micron pyrogen-free filter). The described oligonucleotides may be partially or fully substituted with any of a broad variety of chemical groups or linkages including, but not limited to: phosphoramidates; phosphorothioates; alkyl phosphonates; 2'-O-methyls; 2'-modified RNAs; morpholino groups; phosphate esters; propyne groups; or chimerics of any combination of the above groups or other linkages (or analogs thereof).

A variety of standard methods can be used to purify the presently described oligonucleotides. In brief, the oligonucleotides of the present invention can be purified by chromatography on commercially available reverse phase (for example, see the RAININ Instrument Co., Inc. instruction manual for the DYNAMAX®-300A, Pure-DNA reverse-phase columns, 1989, or current updates thereof, herein incorporated by reference) or ion exchange media such as Waters' Protein Pak or Pharmacia's Source Q (see generally, Warren and Vella, 1994, "Analysis and Purification of Synthetic Nucleic Acids by High-Performance Liquid Chromatography", in *Methods in Molecular Biology*, vol. 26; *Protocols for Nucleic Acid Conjugates*, S. Agrawal, Ed., Humana Press, Inc., Totowa, NJ; Aharon *et al.*, 1993, *J. Chrom.* 698:293-301; and Millipore Technical Bulletin, 1992, *Antisense DNA: Synthesis, Purification, and Analysis*). Peak fractions can be combined and the samples concentrated and desalted via alcohol (ethanol, butanol, isopropanol, and isomers and mixtures thereof, etc.) precipitation, reverse phase chromatography, diafiltration, or gel filtration.

An oligonucleotide is considered pure when it has been isolated so as to be substantially free of, *inter alia*, incomplete oligonucleotide products produced during the synthesis of the desired

oligonucleotide. Preferably, a purified oligonucleotide will also be substantially free of contaminants which may hinder or otherwise mask the binding activity of the oligonucleotide. A purified oligonucleotide, after acidification by one of the disclosed methods or by any other method known to those of skill in the art, is a protonated/acidified oligonucleotide that has been isolated so as to be substantially free of, *inter alia*, excess protonating/acidifying agent.

In particular embodiments, the oligonucleotides of the invention are composed of one or more of the following: partially or fully substituted phosphorothioates, phosphonates, phosphate esters, phosphoroamidites, phosphoroamidates, 2'-modified RNAs, 3'-modified RNAs, peptide oligonucleotides, propynes or analogs thereof.

CHEMICAL MODIFICATIONS OF OLIGONUCLEOTIDES OF THE INVENTION

The oligonucleotides of the invention may contain any modification that confers on the molecules greater binding with other nucleic acids, that increases the acid stability and/or increases the nuclease stability of the molecule. This includes oligonucleotides completely derivatized by phosphorothioate linkages, 2'-O-methylphosphodiesters, 2'-O-alkyl, 2'-O-alkyl-n(O-alkyl), 2'-fluoro, 2'-deoxy-erythropentofuranosyl, p-ethoxy oligonucleotides, p-isopropyl oligonucleotides, phosphoramidates, phosphoroamidites, chimeric linkages, carbonates, amines, formacetals, silyls and siloxys, sulfonates, hydrocarbon, amides, ureas and any other backbone modifications, as well as other modifications, which render the oligonucleotides substantially resistant to endogenous nuclease activity. The nucleotides in each oligonucleotides may each contain the same modifications, may contain combinations of these modifications, or may combine these modifications with phosphodiester linkages. Additional methods of rendering oligonucleotides nuclease resistant include, but are not limited to, covalently modifying the purine or pyrimidine bases that comprise the oligonucleotide. For example, bases may be methylated, hydroxymethylated, or otherwise substituted (*e.g.*, glycosylated) such that the oligonucleotides comprising the modified bases are rendered substantially acid and nuclease resistant.

The ring structure of the ribose group of the nucleotides in the modified oligonucleotide may also have an oxygen in the ring structure substituted with N-H, N-R, S and/or methylene.

Although 2'-O-alkyl substituted oligonucleotides exhibit marked acid stability and endonuclease resistance, they are sensitive to 3' exonucleases. In order to enhance the exonuclease resistance of 2'-O-alkyl substituted oligonucleotides, the 3' or 5' and 3' ends of the ribooligonucleotide sequence are preferably attached to an exonuclease blocking function. For example, one or more phosphorothioate nucleotides can be placed at either end of the oligoribonucleotide. Additionally, one or more inverted bases can be placed on either end of the oligoribonucleotide, or one or more alkyls, *e.g.*, butanol-substituted nucleotides or chemical groups, can be placed on one or more ends of the oligoribonucleotide. Accordingly, a preferred embodiment of the present invention is a protonated/acidified oligonucleotide comprising a oligonucleotide having the following structure:

A-B-C

wherein "B" is a 2'-O-alkyl or 2'-O-alkyl-n(O-alkyl) oligoribonucleotide between about 1 and about 98 bases in length, and "A" and "C" are respective 5' and 3' end blocking groups (*e.g.*, one or more phosphorothioate nucleotides (but typically fewer than six), inverted base linkages, or alkyl, alkenyl, or alkynl groups or substituted nucleotides or 2'-O-alkyl-n(O-alkyl)). A partial list of blocking groups includes inverted bases, dideoxynucleotides, methylphosphates, alkyl groups, aryl groups, cordycepin, cytosine arabanoside, 2'-methoxy, ethoxy nucleotides, phosphoramidates, a peptide linkage, dinitrophenyl group, 2'- or 3'-O-methyl bases with phosphorothioate linkages, 3'-O-methyl bases, fluorescein, cholesterol, biotin, acridine, rhodamine, psoralen, glyceryl, methyl phosphonates, butanol, hexanol, and 3'-O-alkyls. An enzyme-resistant butanol preferably has the structure OH-$CH_2CH_2CH_2CH_2$ (4-hydroxybutyl) which is also referred to as a C4 spacer.

PROTONATED/ACIDIFIED OLIGONUCLEOTIDE COMPOSITIONS

Subsequent to, or during the synthesis and purification steps, protonated/acidified forms of the described end-blocked oligonucleotides can be generated by subjecting the purified, partially purified, or crude oligonucleotides, to a low pH, or acidic, environment. Purified or crude oligonucleotides can be protonated/acidified with acid, including but not limited to, phosphoric acid, nitric acid, hydrochloric acid, acetic acid, etc. For example, acid may be combined with oligonucleotides in solution, or alternatively, the oligonucleotides may be dissolved in an acidic solution. When *in situ* synthesis is desired, the nucleotides may be dissolved in an acidic solution. Excess acid may be removed by chromatography or in some cases by drying the oligonucleotide.

Other procedures to prepare protonated oligonucleotides known to the skilled artisan are equally contemplated to be within the scope of the invention. Once the oligonucleotides of the present invention have been protonated they may be separated from any undesired components like, for example, excess acid. The skilled artisan would know of many ways to separate the oligonucleotides from undesired components. For example, the oligonucleotide solution may be subjected to chromatography following protonation. In a preferred embodiment, the oligonucleotide solution is run over a poly(styrene-divinylbenzene) based resin column (*e.g.*, Hamilton's PRP or Polymer Labs' PLRP) following protonation.

The protonated/acidified oligonucleotides can be used directly, or in a preferred embodiment, processed further to remove any excess acid and salt via precipitation, reverse phase chromatography, diafiltration, or gel filtration. The protonated/acidified oligos can be concentrated by precipitation, lyophilization, solvent evaporation, etc. When suspended in water or saline, the acidified oligonucleotide preparations of the invention typically exhibit a pH between 1 and 4.5 depending upon 1) the level of protonation/acidification, which can be determined by how much acid is used in the acidification process, and 2) the concentration of the oligonucleotide. Alternatively, oligonucleotides can be protonated by passage over a cation exchange column charged with hydrogen ions.

The oligonucleotides of the invention can also be protonated following attachment to the array substrate, *e.g.*, by washing the bound oligonucleotides with an acidic wash followed by a water rinse to neutralize the array before hybridization. Procedures for protonating the oligonucleotides on the array require the oligonucleotides to be bound to an acid-resistant substrate, as will be apparent to one skilled in the art upon reading this disclosure.

EXEMPLARY ARRAY TYPES OF THE SUBJECT INVENTION

A variety of specific array types are also provided by the subject invention. As discussed above, array type refers to the nature of the oligonucleotide compositions present on the array and the types of genes to which the associated compositions correspond. These array types include, but are not limited to: human array; mouse array; developmental array; cancer array; apoptosis array; oncogene and tumor suppressor array; cell cycle gene array; cytokine and cytokine receptor array; growth factor and growth factor receptor array; neuroarrays; and the like.

In certain embodiments of the human array, human genes that may be represented on the subject arrays include: (a) oncogenes and tumor suppressors; (b) cell cycle regulators; (c) stress response proteins; (d) ion channel and transport proteins; (e) intracellular signal transduction modulators and effectors; (f) apoptosis-related proteins; (g) DNA synthesis, repair and recombination proteins; (h) transcription factors and general DNA binding proteins; (i) growth factor and chemokine receptors; (j) interleukin and interferon receptors; (k) hormone receptors; (l) neurotransmitter receptors; (m) cell surface antigens and cell adhesion proteins; (n) growth factors, cytokines and chemokines; (o) interleukins and interferons; (p) hormones; (q) extracellular matrix proteins; (r) cytoskeleton and motility proteins; (s) RNA processing and turnover proteins; (t) post-translational modification, trafficking and targeting proteins; (u) protein turnover; and (v) metabolic pathway proteins.

The arrays of the invention can be used in, among other applications, differential gene expression assays. Thus, arrays are useful in the differential expression analysis of: (a) diseased and

normal tissue, *e.g.*, neoplastic and normal tissue, (b) different tissue or tissue types; (c) developmental stage; (d) response to external or internal stimulus; (e) response to treatment; and the like. The arrays are also useful in broad scale expression screening for drug discovery and research, such as the effect of a particular active agent on the expression pattern of genes in a particular cell, where such information can be used to reveal drug toxicity, carcinogenicity, etc., environmental monitoring, disease research and the like.

HYBRIDIZATION AND DETECTION

Following preparation of the test nucleic acids from the tissue or cell of interest, the test sample is contacted with the array under hybridization conditions, where such conditions can be adjusted, as desired, to provide for an optimum level of specificity in view of the particular assay being performed. In analyzing the differences in the population of labeled test binding agents generated from two or more physiological sources using the arrays described above, each population of labeled test samples are separately contacted to identical arrays or together to the same array under conditions of hybridization, preferably under stringent hybridization conditions (for example, at 50 C or higher and 0.1X SSC (15 mM sodium chloride/01.5 mM sodium citrate)), such that test nucleic acids hybridize to complementary oligonucleotides on the substrate surface.

Where all of the test nucleic acids comprise the same label, different arrays can be employed for each physiological source. Preferably, the same array can be employed sequentially for each physiological source, with test samples removed from the array as described below. Alternatively, where the labels of the test nucleic acids are different and distinguishable for each of the different physiological sources being assayed, the opportunity arises to use the same array at the same time for each of the different test populations. Alternatively, where the labels of the test nucleic acids are different and distinguishable for each of the different physiological sources being assayed, the opportunity arises to use the same array at the same time for each of the different test populations. Examples of distinguishable labels are well known in the art and include: two or more different

emission wavelength fluorescent dyes, like Cy3 and Cy5, two or more isotopes with different energies of emission, like ^{32}P and ^{33}P, labels which generate signals under different treatment conditions, like temperature, pH, treatment by additional chemical agents, etc., or generate signals at different time points after treatment. Using one or more enzymes for signal generation allows for the use of an even greater variety of distinguishable labels, based on different substrate specificity of enzymes (*e.g.*, alkaline phosphatase/peroxidase).

Following hybridization, non-hybridized labeled nucleic acid is removed from the support surface, conveniently by washing, generating a pattern of hybridized oligonucleotide on the substrate surface. A variety of wash solutions are known to those of skill in the art and may be used. The resultant hybridization patterns of labeled, hybridized oligonucleotides may be visualized or detected in a variety of ways, with the particular manner of detection being chosen based on the particular label of the test nucleic acid, where representative detection means include scintillation counting, autoradiography, fluorescence measurement, colorimetric measurement, light emission measurement and the like.

Following detection or visualization, the hybridization patterns may be compared to identify differences between the patterns. Where arrays in which each of the different oligonucleotides corresponds to a known gene are employed, any discrepancies can be related to a differential expression of a particular gene in the physiological sources being compared.

CLEARING OF TEST NUCLEIC ACIDS FROM ARRAY

Following binding and visualization of a test sample on an array, the array may be treated to remove the bound test nucleic acids. The associated nucleic acid compositions remain intact following treatment, allowing reuse of the treated array. The array of the invention substantially retains its binding capabilities, and any differences in binding ability may be determined using control sequences associated on the array. Preferably, the array of the invention retains at least 75% of its binding capabilities, more preferably the array retains at least 85% of its binding

capabilities, and even more preferably the array of the invention retains at least 95% of its binding capabilities.

Arrays with associated protonated/acidified oligonucleotide compositions can be exposed to a low pH environment, *e.g.*, pH from 0.5 -4.5, which results in the degradation of non-modified nucleic acids. Following the treatment, the arrays of the invention are rinsed to remove any unwanted test nucleic acid fragments, residual label and the like, and the arrays are prepared for reuse.

After detection of the array plus sample is complete, the array may be regenerated by removal and/or degradation of the test sample. For example, a two hour incubation of the sample-bound array in an acid solution at pH 1.5, 39 C, results in complete loss of a full-length unmodified 14-mer oligonucleotide. Under these conditions the bound array oligonucleotides of the invention maintain full length structural integrity. Following the acid incubation, a variety of wash conditions may be used to clear the test sample from the probe array. For example, increased temperature incubation of a low salt wash solution would result in the dissociation of short test fragments from the array. Alternatively, a chemical denaturant (*e.g.*, urea) could be used as a wash to remove the test sample. Additional steps, such as an alkaline solution rinse may also be added to the protocol to speed up the cycle time for regeneration.

The above-described washes and rinses can be avoided if the acid incubation is increased resulting in almost complete degradation of the test sample under conditions where the array probe maintains its integrity. Actual incubation times required will vary somewhat from array type to array type, and may be shorter than those given below. As a consequence of the degradation of the test sample the array probe/test sample hybrids become unstable under experimental conditions and may be removed using rinses of the hybridization or stringent wash buffer.

Exemplary clearing conditions for use with the arrays of the invention are:

(1) Incubation of the bound array with pH 1-2 acid solution, 8 hours at 39 C. Follow with three rinses at 39 C with stringent wash buffer, 0.1 X SSC pH 7.0, and two rinses with

hybridization buffer, pH approximately 7.0. These two solutions are for removal of degraded sample and the regeneration of the substrate array and hence do not require a low pH. Array may then be reused.

(2) Incubation of the bound array with pH 1-2 acid solution, 4 hours at 39 C. Follow with three 15 minute rinses at 39 C with 8.0 molar urea. Rinse once with stringent wash buffer, and twice with hybridization buffer. Array can be reused at this point.

(3) Incubation of the bound array with pH 1-2 acid solution, 4 hours at 39 C. Rinse twice at 39 C with stringent wash buffer. Incubate 20 minutes in 60 C stringent wash buffer, and rinse twice more with 60 C stringent wash buffer. Rinse twice with hybridization buffer. Array can be reused at this point.

(4) Incubation of the bound array with pH 1-2 acid solution, 4 hours at 39 C. Rinse twice with stringent wash buffer. Wash twice with 39 C alkaline solution for 15 minutes followed by two washes with stringent wash buffer. Incubate 20 minutes in 60 C stringent wash buffer. Rinse twice more with 60 C stringent wash buffer, and twice with hybridization buffer. Array can be reused at this point.

(5) Incubation of the bound array with nuclease (actual conditions vary with nuclease type) at 37 C for 1 hour. Wash twice with protein denaturing solution for 20 minutes. Rinse twice with stringent wash buffer. Incubate 20 minutes in 60 C stringent wash buffer. Rinse twice with 60 C stringent wash buffer. Rinse twice with hybridization buffer. Array can be reused at this point.

Following treatment, the associated acid stable oligonucleotides of the array remain 1) associated to the substrate surface; 2) structurally intact; and 3) capable of binding with another test binding partner.

In addition, as an alternative way, arrays with associated oligonucleotides characterized as nuclease resistant may be treated with a nuclease to remove bound test nucleic acids and label. The nuclease used can be chosen depending on the nature of the binding between the associated

oligonucleotide and the molecules of the test sample and the attachment of the oligonucleotide to the array. For example, if the associated oligonucleotides are end-blocked oligonucleotides, and the test sample is comprised of mRNA molecules, then the appropriate nuclease would be one that recognizes RNA-DNA hybrids, *e.g.*, Ribonuclease H. In another example, if the associated oligonucleotides are end-blocked oligonucleotides, and the test sample is comprised of cDNA molecules, then the appropriate nuclease would be one that recognizes double stranded DNA complexes, *e.g.*, Deoxyribonuclease I or II, and Exodeoxyribonuclease III or V. In yet another example, if the associated oligonucleotides are end-blocked cRNA and the test sample is comprised of mRNA, the appropriate nuclease is one that recognizes RNA-RNA hybrids, such as micrococcal nuclease. Similarly, nucleases that are 5' or 3' specific may be chosen depending on the attachment site of the oligonucleotide to the array. Since the oligonucleotides of this embodiment of the invention are nuclease-resistant, the test samples will be specifically targeted and degraded by the nuclease.

Actual choice of regeneration conditions should take into consideration the type of substrate, the type of attachment of probe to substrate, test sample type, and whether there are clearing time constraints. In cases where the substrate is acid sensitive it would be more advantageous to use nuclease digestion to remove the test sample from the array. Such modifications would be well within the skill of one in the art upon reading the present disclosure and description of the subject arrays.

KITS HAVING ARRAYS OF PRESENT INVENTION

Also covered are kits for performing analyte binding assays using the arrays of the present invention. Such kits according to the subject invention will at least comprise the arrays of the invention having associated modified oligonucleotides. Kits also preferably comprise an agent for removal of test binding agents, *e.g.*, a solution with low pH and/or with nuclease activity. The kits may further comprise one or more additional reagents employed in the various methods, such as:

1) primers for generating test nucleic acids; 2) dNTPs and/or rNTPs (either premixed or separate), optionally with one or more uniquely labeled dNTPs and/or rNTPs (*e.g.*, biotinylated or Cy3 or Cy5 tagged dNTPs); 3) post synthesis labeling reagents, such as chemically active derivatives of fluorescent dyes; 4) enzymes, such as reverse transcriptases, DNA polymerases, and the like; 5) various buffer mediums, *e.g.*, hybridization and washing buffers; 6) labeled probe purification reagents and components, like spin columns, etc.; and 7) signal generation and detection reagents, *e.g.*, streptavidin-alkaline phosphatase conjugate, chemifluorescent or chemiluminescent substrate, and the like.

EXAMPLES

The present invention and its particular embodiments are illustrated in the following examples. The examples are not intended to limit the scope of this invention but are presented to illustrate and support the claims of this present invention.

EXAMPLE 1: Synthesis, Purification and Protonation/Acidification of Nucleic Acids

Oligonucleotides were synthesized using commercial phosphoramidites on commercially purchased DNA synthesizers from <1 uM to >1mM scales using standard phosphoramidite chemistry and methods that are well known in the art, such as, for example, those disclosed in Stec *et al.*, *J. Am. Chem. Soc.* 106:6077-6089 (1984), Stec *et al.*, *J. Org. Chem.* 50(20):3908-3913 (1985), Stec *et al.*, *J. Chromatog.* 326:263-280 (1985), LaPlanche *et al.*, *Nuc. Acid. Res.* 14(22):9081-9093 (1986), and Fasman, *Practical Handbook of Biochemistry and Molecular Biology*, 1989, CRC Press, Boca Raton, FL, herein incorporated by reference.

Oligonucleotides were deprotected following phosphoramidite manufacturer's protocols. Unpurified oligonucleotides were either dried down under vacuum or precipitated and then dried. Sodium salts of oligonucleotides were prepared using the commercially available DNA-Mate

(Barkosigan Inc.) reagents or conventional techniques such as commercially available exchange resin, *e.g.*, Dowex, or by addition of sodium salts followed by precipitation, diafiltration, or gel filtration, etc.

A variety of standard methods were used to purify/produce the presently described oligonucleotides. In brief, oligonucleotides were purified by chromatography and protonated on commercially available reverse phase (for example, see the RAININ Instrument Co., Inc. instruction manual for the DYNAMAX®-300A, Pure-DNA reverse-phase columns, 1989, or current updates thereof, herein incorporated by reference) or ion exchange media such as Waters' Protein Pak or Pharmacia's Source Q (see generally Warren and Vella, 1994, "Analysis and Purification of Synthetic Nucleic Acids by High-Performance Liquid Chromatography", in *Methods in Molecular Biology*, vol. 26; *Protocols for Nucleic Acid Conjugates*, S. Agrawal, Ed. Humana Press, Inc., Totowa, NJ; Aharon *et al.*, 1993, *J. Chrom.* 698:293-301; and Millipore Technical Bulletin, 1992, *Antisense DNA: Synthesis, Purification, and Analysis*). Peak fractions were combined and the samples were concentrated and desalted via alcohol (ethanol, butanol, isopropanol, and isomers and mixtures thereof, etc.) precipitation, reverse phase chromatography, diafiltration, or gel filtration or size-exclusion chromatography.

Subsequently, or during the above steps, protonated/acidified forms of the described oligonucleotides can be generated by subjecting the purified, or partially purified, or crude oligonucleotides, to a low pH, or acidic, environment. Purified or crude oligonucleotides were protonated/acidified with acid, including but not limited to, phosphoric acid, nitric acid, hydrochloric acid, acetic acid, etc.

Pooled fractions of a SAX-purified oligonucleotide (at approximately 2-25 A_{260} per ml) were pumped into a poly(styrene-divinylbenzene) based column, such as Polymer Labs' PLRP or Hamilton's PRP-1 or PRP-3. This was followed immediately with an excess of dilute acid (*e.g.*, 100mM HCl) until the eluent was acidic. The column was then washed with purified water (no salt or buffers) until the conductivity of the eluent returned to essentially background levels and

background pH. The oligonucleotides were then dried down in a commercially available vacuum evaporator. Alternatively, the oligonucleotides were suspended in dilute acid and either chromatographed over the PRP or similar poly(styrene-divinylbenzene) based columns as described above, or chromatographed over a size exclusion column or gel filtration column (*e.g.*, BioRad P2 or P4) using water as solvent. Alternatively, a desalted oligonucleotide may be dissolved in alkaline salt solution (*e.g.*, 0.4 M NaCl and pH 12, 25 mM NaOH), run on a PRP or similar poly(styrene-divinylbenzene) based column, washed with acid followed by water, and then eluted, as described above.

Alternatively, a oligonucleotide may be chromatographed over a cation exchange column that is in the H^+ form, collected and dried down as described above.

Oligonucleotides were also acidified by adding an acid, *e.g.*, HCl (0.1 N) directly to a oligonucleotide solution (approximately 300 A_{260} per ml) until the pH of the solution reached pH 1 to pH 3. The acidified oligonucleotides can then be run over an acid stable size exclusion column such as a BioRad P-gel column.

Lyophilized or dried-down preparations of oligonucleotides were dissolved in pyrogen-free, sterile, physiological saline (i.e., 0.85% saline), sterile Sigma water, and filtered through a 0.45 micron Gelman filter.

When suspended in water or saline, the oligonucleotide preparations typically exhibited a pH between 1 and 4.5 depending upon the level of protonation/acidification, which is determined by how much acid is used in the acidification process.

EXAMPLE 2: Stability of Modified Oligonucleotide Duplexes

The stability of duplexes having 2'-substituted nucleotides versus duplexes without such modification was tested by examining the T_m of these complexes. 4 μM each of 20-mer oligonucleotide (5' - ggt ggt tcc tcc tca gtc gg -3'; SEQ ID NO:1) and its complement (5'- ccg act

gag aag gaa cca cc -3') were bound in a solution of 50 mM NaCl, 10 mM PO4 buffer, pH 7.4. Each of the nucleotides of the oligonucleotide had the same 2' group. Following binding, the melting temperature was determined as described. (See L.L.Cummins et al, Nucleic Acids Research 23:2019-2024 (1995).

Results were as follows:

SEQ ID NO:1		SEQ ID NO:2	T_m
Regular RNA	and	Regular DNA	66 C
Regular RNA	and	2'-O-methyl	79 C
Regular DNA	and	p-ethoxy DNA	55 C
Regular RNA	and	p-ethoxy RNA	56 C
Regular RNA	and	p-ethoxy 2'-O-methyl	71 C

The duplexes with the 2'-O-methyl substitutions display a significantly increased Tm compared to RNA or DNA with a 2' H or 2' OH, respectively. RNA or DNA with propyl or fluoro substitutions at the 2' position display an even higher T_m than does the 2'-O-methyl.

EXAMPLE 3: Acid Stability of the Oligonucleotides of the Invention

Homopolymers of 2'-O-methyl A, C, G, and U twelve bases long, were synthesized with 3' and 5' inverted T-blocked ends. They were purified, desalted, lyophilized, and dissolved at 300 A_{260} per ml in sterile water. Samples were removed and diluted 1 to 4 with either 0.1 N HCl or 1.0 N HCl to give final pHs of approximately 1 and 0, respectively, and placed in a heat block at 39 C. Aliquots were taken at 0, 2, 4 and 24 hours, diluted 1:20 into a solution of 0.025 M NaOH and 0.03 M NaCl, stored at -20 C until being run on an analytical HPLC under strongly denaturing conditions on an anion exchange column.

		% Full Length			
Homopolymer	pH	0 hr	2 hr	4 hr	24 hr
A	1	99	99	99	99
C	1	99	99	99	96
G	1	96	98	98	98
U	1	97	--	97	97
A	0	99	99	99	99
C	0	99	99	98	97
G	0	96	97	97	89
U	0	97	--	97	96

It was evident that there is essentially no degradation at pH 1 and 39 C and only slight degradation over 24 hours at pH 0 and 39 C.

EXAMPLE 4: Acid Stability of the Oligonucleotides of the Invention

A 14 mer heteropolymer was synthesized as a regular phosphodiester DNA (O), a phosphorothioate DNA (S), an unblocked 2'-O-methyl RNA (2'om), a 2'-O-methyl RNA with 3' and 5' butanol blocked ends (B2'om), and a phosphorothioate chimera having four 2'-O-methyl phosphorothioate bases on either side of 6 interior phosphorothioate DNA bases (SD). They were purified, desalted, lyophilized, and dissolved at 300 A_{260} per ml in sterile water. Samples were

removed and diluted 1 to 4 with 0.1 N HCl to give a final pH of approximately 1.5, and placed in a heat block at 39 C. Aliquots were taken at the times indicated and diluted 1:20 into a solution of 0.025 M NaOH and 0.03 M NaCl, and were run on an analytical HPLC under strongly denaturing conditions on an anion exchange column. Initially all but the end-blocked 2'-O-methyl RNA solutions became cloudy upon addition of the HCl. Upon heating, both the phosphodiester DNA and the unblocked 2'-O-methyl RNA became clear. The two oligonucleotides with phosphorothioate linkages appeared cloudy until about 2 hours when they slowly began to clear as they decomposed.

	% Full Length											
Oligo	**0 hr**	**0.5 hr**	**1.0 hr**	**2 hr**	**4 hr**	**6 hr**	**1 d**	**2 d**	**3 d**	**5 d**	**10 d**	**20 d**
O	99	38	10	0	0	0	0	--	--	--	--	--
S	95	65	29	1	0	0	0		--	--	--	--
SD	97	83	70	49	0	0	0	--	--	--	--	--
2'om	99	99	99	99	98	98	98	96	94	94	87	80
B2'om	100	100	100	100	99	99	98	97	97	95	90	81

The 2'-O-methyl oligonucleotides, both unblocked and blocked, are far more stable than the corresponding phosphodiester, phosphorothioate, or a mixed 2'-O-methyl phosphorothioate structure that Agrawal *et al.* recommended to increase bioavailability.

EXAMPLE 5: Direct Synthesis on a Two-dimensional Substrate Using Photoremovable Groups

Modified oligonucleotides having predetermined polynucleotide sequences are synthesized on a solid substrate in the form of a spatially defined array, wherein the sequences of an oligonucleotide are positionally determined.

Using the present method, the formation of modified oligonucleotides on the substrate requires the stepwise attachment of a nucleotide to a substrate-bound growing oligomer. In order to prevent unwanted polymerization of the monomeric nucleotide under the reaction conditions, protection of the 5'-hydroxyl group of the nucleotide is required. After the monomer is coupled to the end of the oligomer, the 5'-hydroxyl protecting group is removed, and another 2'-modified nucleotide is coupled to the chain. This cycle of coupling and deprotecting is continued for each nucleotide in the oligomer sequence. See Gait, "Oligonucleotide Synthesis: A Practical Approach" 1984, IRL Press, London, incorporated herein by reference for all purposes. The use of a photoremovable protecting group allows removal, via patterned irradiation, of selected portions of the substrate surface during the deprotection cycle of the solid phase synthesis. This selectively allows spatial control of the synthesis-the next 2'-modified nucleotide is coupled only to the irradiated areas.

2'-modified oligonucleotide synthesis takes place by coupling an activated phosphorous derivative on the 3'-hydroxyl group of each 2'-modified nucleotide with the 5'-hydroxyl group of an oligomer bound to a solid support. A photoremovable protecting group, MeNV, is attached to an activated 2'-modified nucleotide on the 5'-hydroxyl group.

Following synthesis, the substrate is irradiated to remove the photoremovable protecting groups and create regions having free reactive moieties and side products resulting from the protecting group. Removal of the protecting group is accomplished by irradiation to liberate the reactive group and degradation products derived from the protecting group.

EXAMPLE 6: Direct Synthesis on a Two-dimensional Substrate Using Controlled Introduction

A modified oligonucleotide array is synthesized on site using a technique that allows controlled introduction of individual nucleotides to specific regions on an array surface. The array is produced by systematically laying down each of the four modified bases in a predetermined

pattern. Such a technique is described in U.S. Pat. No. 5, 700,637. One such method employs the use of a printer, such as an ink jet printer, to perform such directed synthesis.

Glass slides are reacted with a chemical linker to provide binding sites for attaching the immobilized species. The surface of the slide is coated with the linker solution. 2' modified phosphorotioate oligonucleotides are synthesized by placing the bases in a predetermined pattern. The initial modified nucleotides are then systematically placed on the linker-coated surface as a plurality of circular regions on the surface, each region having the modified nucleotide specific to the sequence of the desired oligonucleotide for that region, *e.g.* a modified G, A, T, U, C or even I when degeneracy is required. This first immobilized species act as a basis for the attachment of other modified nucleotides in the formation of specific modified oligonucleotides at each region.

While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

CLAIMS

That which is claimed is:

1. A method for detecting nucleic acid sequences in two or more collections of nucleic acid molecules, the method comprising:

(a) providing an array of modified polynucleotides bound to a solid surface, each said modified polynucleotide comprising a determinable nucleic acid;

(b) contacting the array of modified polynucleotides with a first collection of labeled nucleic acid comprising a sequence substantially complementary to a nucleic acid of said array, and

detecting hybridization of the first collection of labeled complementary nucleic acids to nucleic acids of said arrays;

(c) removing said hybridized nucleic acids;

(d) contacting said array with a second collection of labeled nucleic acid comprising a sequence substantially complementary to a modified polynucleotide of said array; and

(c) detecting hybridization of the first and second labeled complementary nucleic acids to nucleic acids of said arrays;

wherein the modified oligonucleotides are characterized by a pH stability of at least one hour at 37 C at a pH in a range of about 0.5 to 6 and a nuclease resistance of at least twice that of a naturally occurring oligonucleotide having the same sequence and number of bases.

2. The method of claim 1, wherein the first and second collections of nucleic acids are differentially labeled.

3. The method of claim 1, wherein the first and second collections of nucleic acids comprise the same detectable label.

4. The method of claim 1, wherein the step of removing said hybridized nucleic acids comprises incubation of the array with pH 1-2 acid solution.

5. The method of claim 1, wherein the step of removing said hybridized nucleic acids comprises incubation incubation of the array with nuclease.

6. A method of identifying nucleotide differences between the sequence of a target nucleic acid and the sequence of a reference nucleic acid comprising:

a) providing a substrate comprising different modified polynucleotide probes of known sequence at known locations;

b) contacting the target nucleic acid with the modified polynucleotide probes attached to the substrate under conditions for high specificity complementary hybridization;

c) determining which modified polynucleotide probes have hybridized with the target nucleic acid;

d) removing hybridized target nucleic acid from the polynucleotide probes;

e) contacting the reference nucleic acid with the modified polynucleotide probes attached to the substrate under conditions for high specificity complementary hybridization; and

f) comparing the sequence of the reference nucleic acid with the sequences of the modified polynucleotide probes that have hybridized with the target nucleic acid and to identify the nucleotide differences between the sequence of the target nucleic acid and the sequence of the reference nucleic acid.

7. The method of claim 6, wherein each of the different modified polynucleotide probes is attached to the surface of the substrate in a different predefined region.

8. The method of claim 7, wherein each of the modified polynucleotide probes in a predefined region has a different determinable sequence, and further wherein each probe is at least 4 nucleotides in length.

further wherein the modified oligonucleotides are characterized by a characteristic selected from the group consisting of (a) a binding affinity of at least about 1.25 times that of a corresponding, non-modified oligonucleotide, (b) a pH stability of at least one hour at 37 C at a pH in a range of about 0.5 to 10; and (c) a nuclease resistance of at least twice that of a naturally occurring oligonucleotide having the same sequence and number of bases.

METHOD FOR DETECTING NUCLEIC ACID SEQUENCES

ABSTRACT OF THE DISCLOSURE

A method for detecting nucleic acid sequences in two or more collections of nucleic acid molecules, the method comprising:

(a) providing an array of modified polynucleotides bound to a solid surface, each said modified polynucleotide comprising a determinable nucleic acid;

(b) contacting the array of modified polynucleotides with:

(i) a first collection of labeled nucleic acid comprising a sequence substantially complementary to a nucleic acid of said array, and

(ii) at least a second collection of labeled nucleic acid comprising a sequence substantially complementary to a modified polynucleotide of said array;

wherein the first and second labels are distinguishable from each other; and

(c) detecting hybridization of the first and second labeled complementary nucleic acids to nucleic acids of said arrays;

wherein the modified oligonucleotides are characterized by a characteristic selected from the group consisting of (a) a binding affinity of at least about 1.25 times that of a corresponding, non-modified oligonucleotide, (b) a pH stability of at least one hour at 37 C at a pH in a range of about 0.5 to 10; and (c) a nuclease resistance of at least twice that of a naturally occurring oligonucleotide having the same sequence and number of bases.

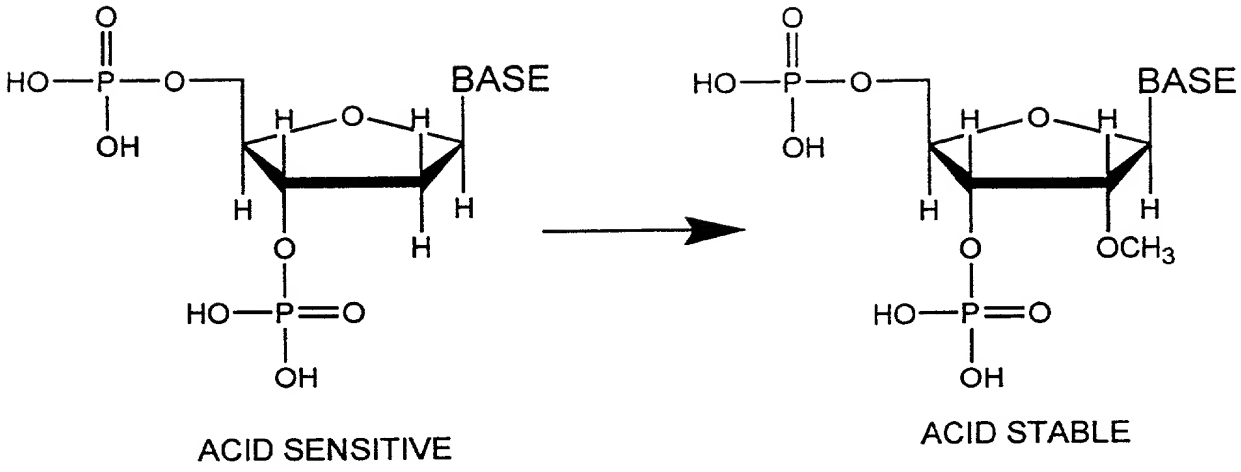

HO
P
O
OH
BASE
H
O
H
H
H
H
O
HO
P
O
OH
ACID SENSITIVE
HO
P
O
OH
BASE
H
O
H
H
H
OCH3
O
HO
P
O
OH
ACID STABLE

Figure 1

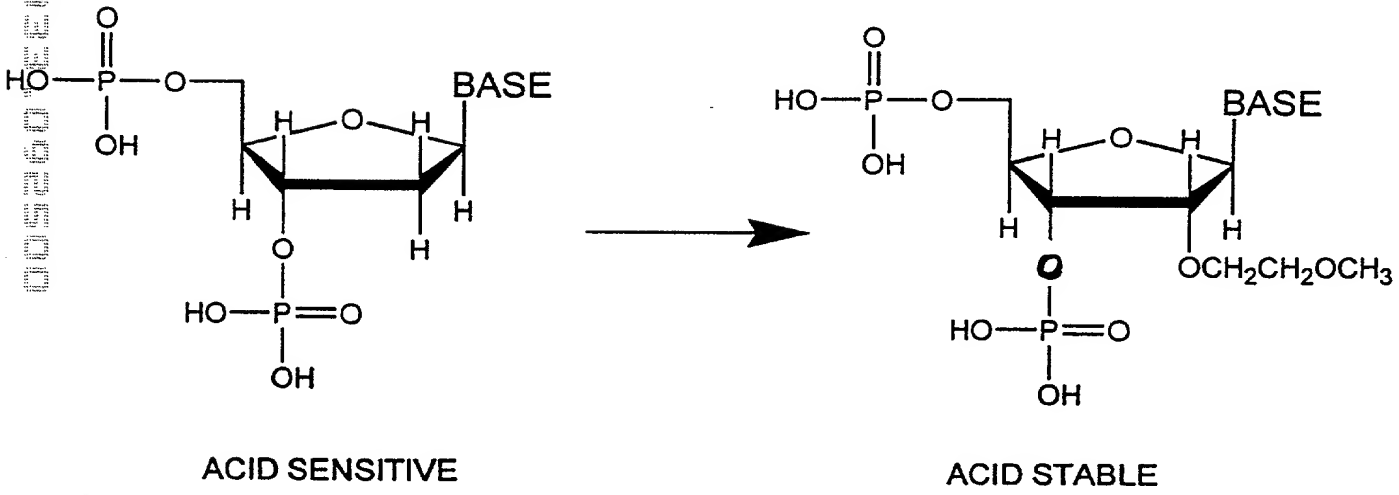
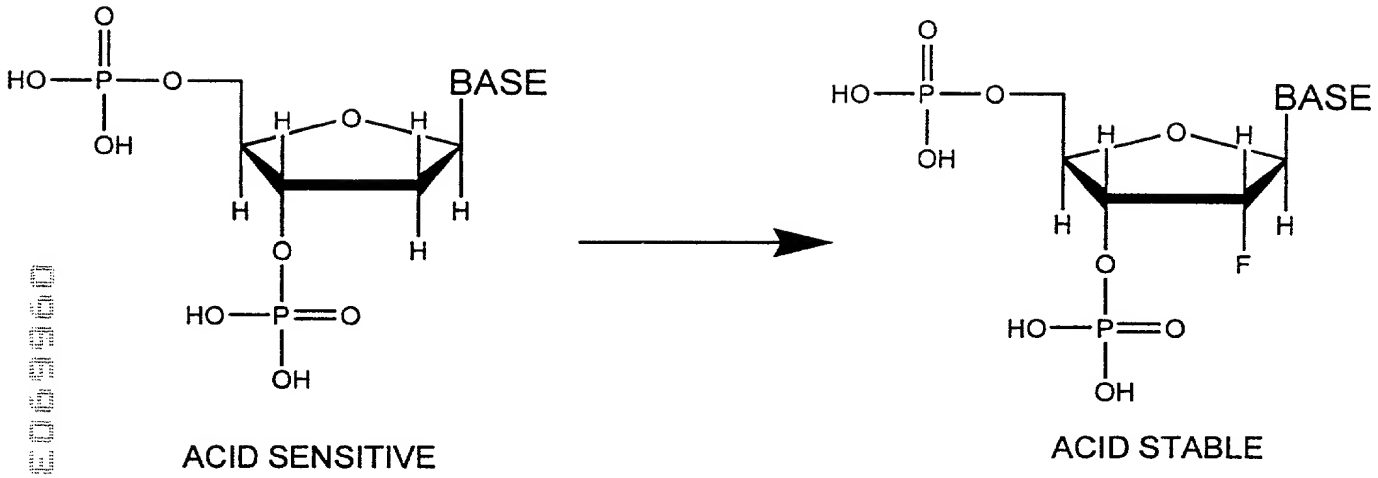

HO
P
O
OH
BASE
H
O
HO
P
O
OH
ACID SENSITIVE
HO
P
O
OH
BASE
H
O
H
OCH2CH2OCH3
HO
P
O
OH
ACID STABLE

Figure 2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P
O
OH
BASE
H
O
H
H
H
O
HO
P
O
OH
ACID SENSITIVE
HO
P
O
OH
BASE
H
O
H
H
F
O
HO
P
O
OH
ACID STABLE

Figure 3

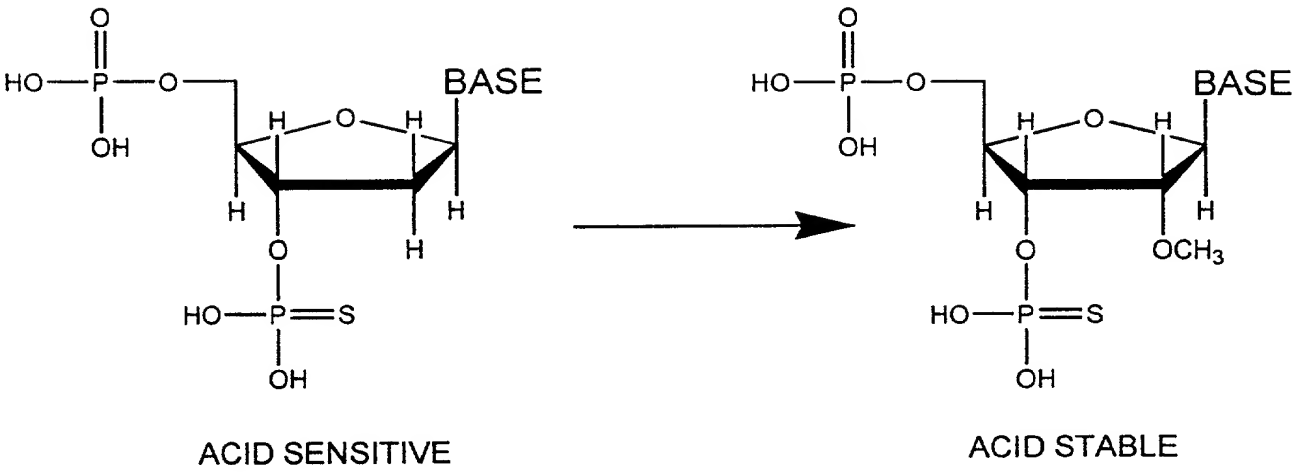

Figure 4

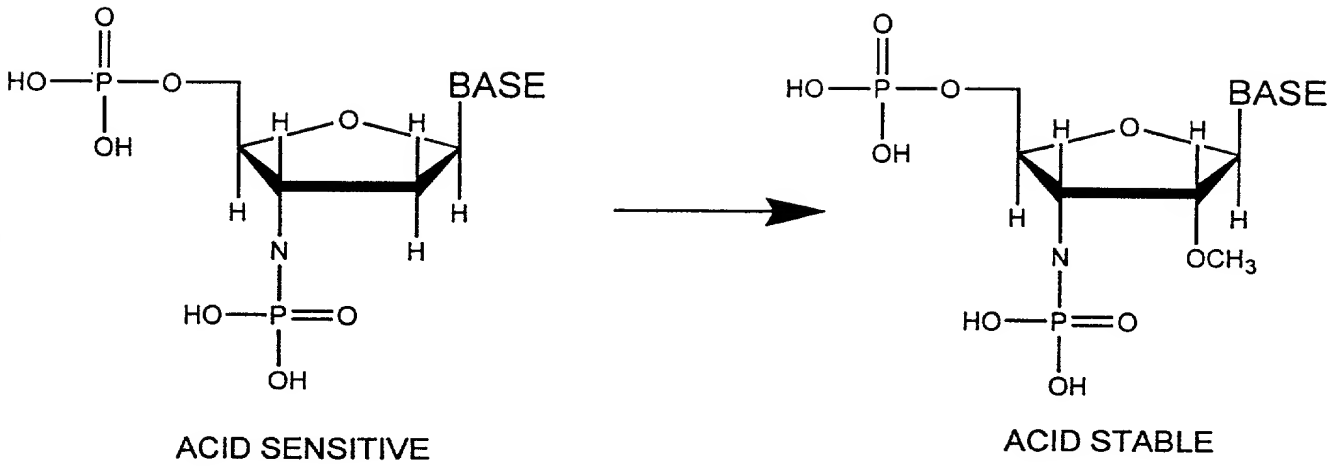
HO
P
O
OH
BASE
H
O
H
N
HO
P
O
OH
ACID SENSITIVE
HO
P
O
OH
BASE
H
O
H
N
OCH3
HO
P
O
OH
ACID STABLE

Figure 5

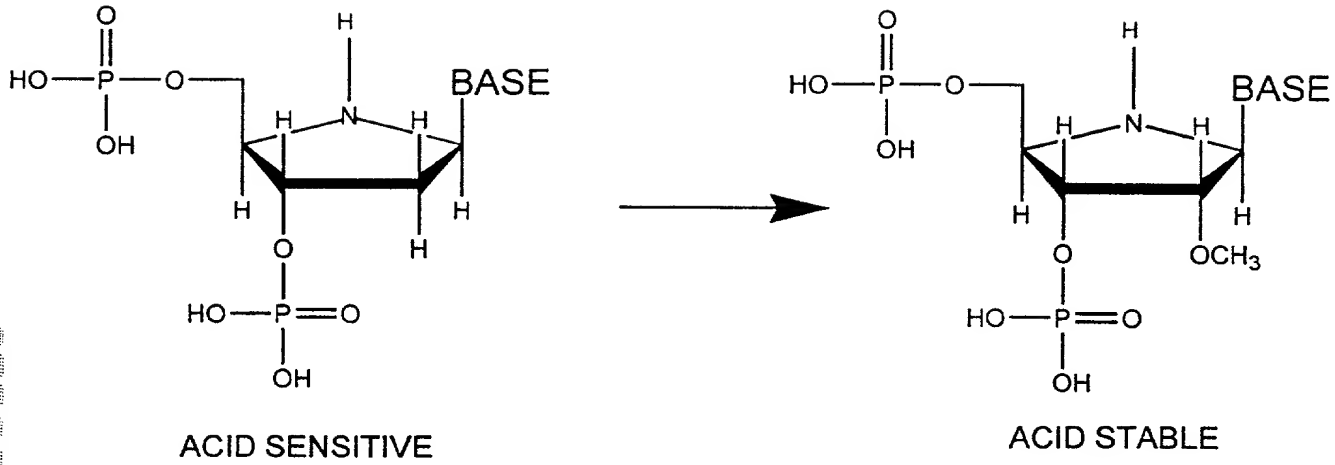

Figure 6

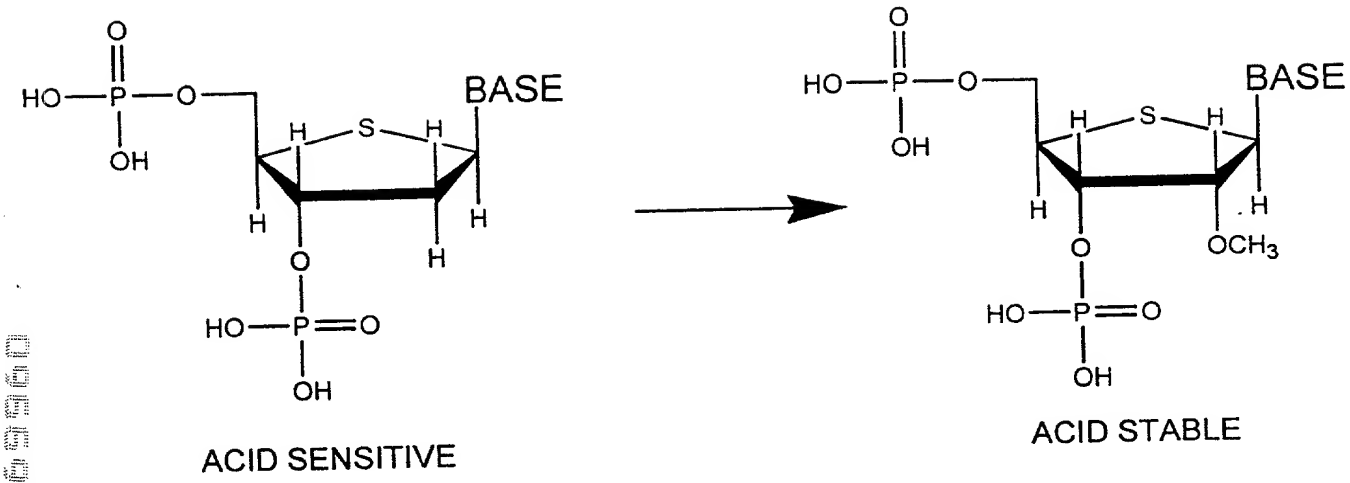
HO
P
O
OH
BASE
H
S
H
H
H
H
O
HO
P
O
OH
ACID SENSITIVE
HO
P
O
OH
BASE
H
S
H
H
H
O
OCH3
HO
P
O
OH
ACID STABLE

Figure 7

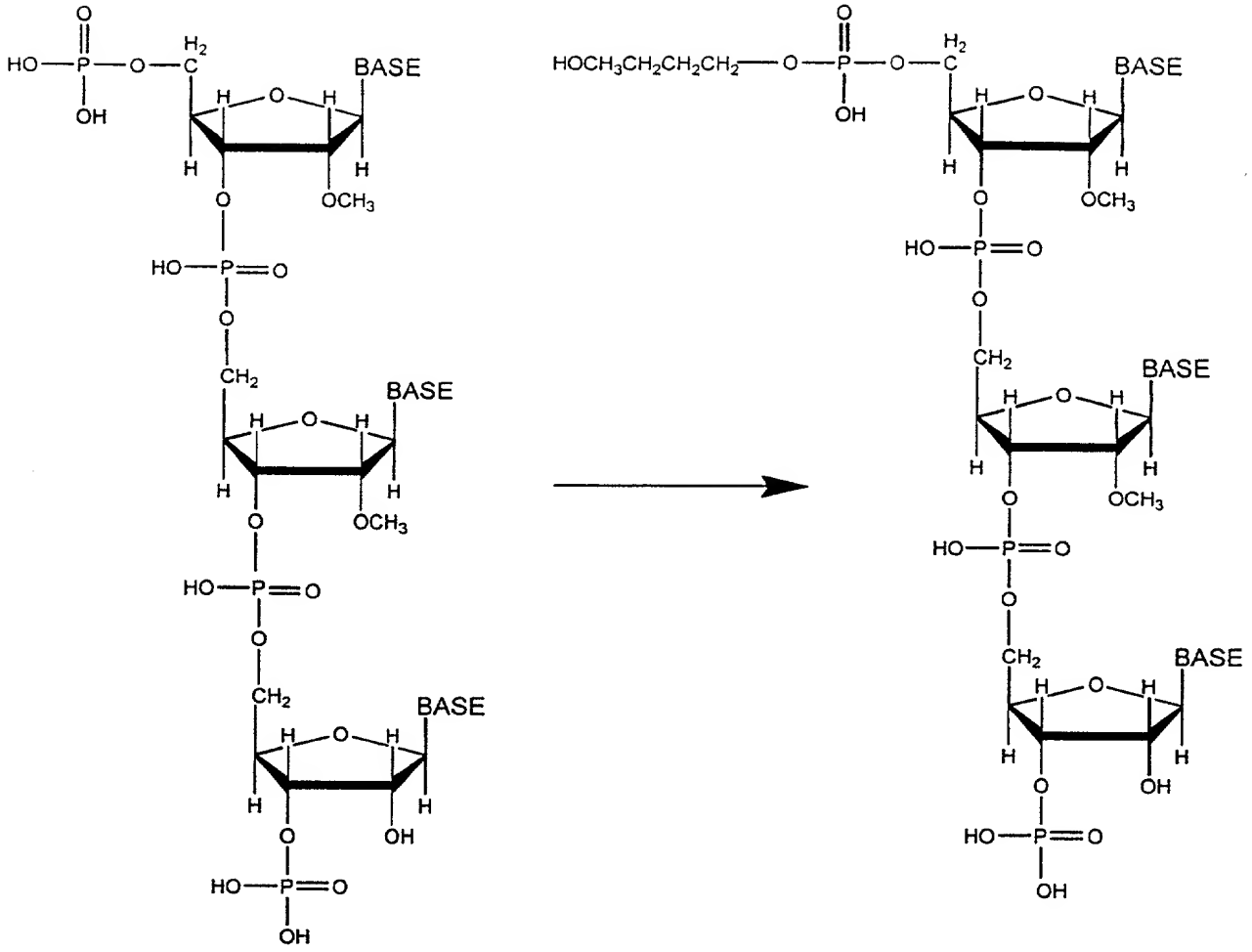
BASE
OCH3
OH
HOCH3CH2CH2CH2
ACID STABLE
ACID STABLE, END-BLOCKED
5' end
3' Butanol Blocking Group

Figure 8

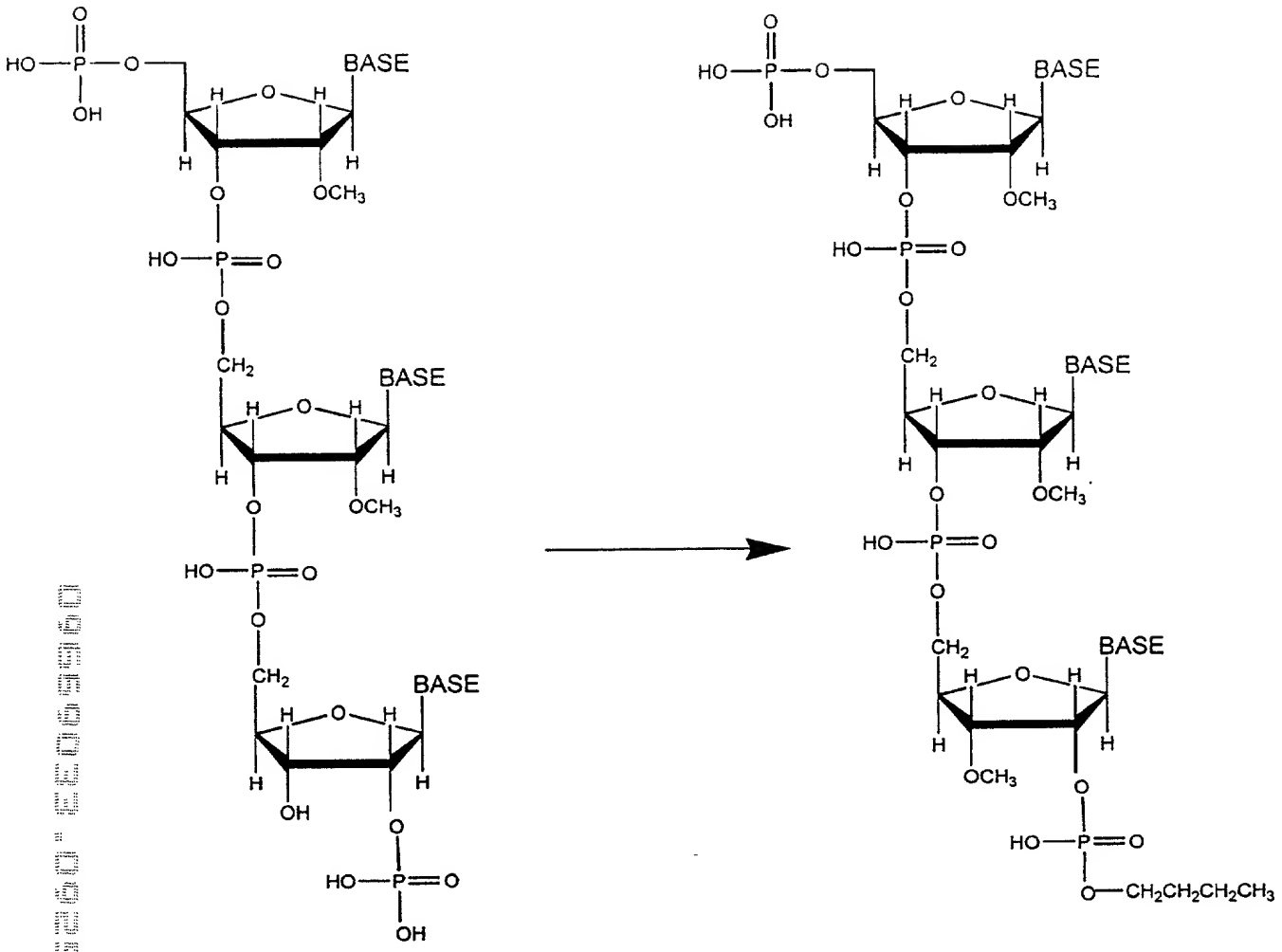
BASE
OCH3
CH2
OH
ACID STABLE
ACID STABLE, END-BLOCKED
3' end
Butyl Blocking Group

Figure 9

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)		
	Attorney Docket Number	OLIG-017
	First Named Inventor	Roderic M.K. Dale
	COMPLETE IF KNOWN	
	Application Number	09/385,796
	Filing Date	August 30, 1999
	Group Art Unit	1643
	Examiner Name	To Be Assigned

__ Declaration Submitted with Initial Filing OR __ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16(e)) required)

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHOD FOR DETECTING NUCLEIC ACID SEQUENCES

the specification of which:

__ is attached hereto

OR

_X_ was filed on August 30, 1999 as United States Application Number or PCT International Application Number 09/385,796 and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined by 37 CFR 1.56.

Insofar as the subject matter of each of the claims of this application are not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designating at least one country other than the United States of America, listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) having a filing date before that of the application(s) of which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
			__	__	__
			__	__	__
			__	__	__
			__	__	__

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application(s) designating the United States of America, listed below.

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number *(if applicable)*

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Name	Registration Number	Name	Registration Number
Karl Bozicevic	28,807	Bret E. Field	37,620
Carol L. Francis	36,513	Pamela J. Sherwood	36,677
Dianna L. DeVore	42,484	Paula A. Borden	42,344

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City, State, Zip	Palo Alto, California 94301				
Country	U.S.A.	**Telephone**	650-327-3400	**Facsimile**	650-327-3231

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor:

Given Name (first and middle [if any])	Family Name or Surname
RODERIC M.K.	DALE

Inventor's Signature	Roderic M.K. Dale					**Date**	10/12/99
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Post Office Address	26761 SW 45th Drive						
City	Wilsonville	**State**	OR	**Zip**	97070	**Country**	U.S.A.